

THE ROLE OF BACTERIAL ROOT ENDOPHYTES IN TOMATO GROWTH AND DEVELOPMENT

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A Dissertation

*Submitted to the Faculty of Purdue University
In Partial Fulfillment of the Requirements for the degree of*

Doctor of Philosophy



Department of Botany & Plant Pathology
West Lafayette, Indiana
December 2022

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Dedicated to my parents, for nurturing me the love for life and nature.

ACKNOWLEDGMENTS

First, I would like to express my profound appreciation to my advisor Dr. Anjali Iyer-Pascuzzi. I am proud to be your student. Throughout my short years in graduate school, I am privileged to witness and learn from you. You lead by example with a foundation of dignity, hard work, and kindness. You always encourage me to reach out of my comfort zone and become a better version of myself every day. It was evidenced when I was the first student in the department to do a 7-month industry internship. That opportunity would only happen with you advocating for me in my scientific training and professional development. Thank you from the bottom of my heart for being my mentor.

I want to give a special appreciation to my committee members: Drs. Zhixiang Chen, Lori Hoagland, and Tesfaye Mengiste, for your guidance, insights, and expert suggestion. I am grateful for your efforts in training me to think outside the box, be creative, and sharpen my scientific analytical skills. I thank the Center for Plant Biology-Purdue University for supporting my Ph.D and funding my travel to national conferences.

I am also thankful for the friendship and support from all of my labmates, both past and present, whom I was so lucky to meet and befriend with: Lizzie, Denise, Kathe, Rachel, Beka, Abbie, Sana, and Chloe. All of you have a special place in my heart and reasons for the success of my graduate career. Lizzie, thank you for being my mentor and passing me your skills and passion for plants and science. Denise, I thank you for your patience, love, dedication to helping me succeed, and for welcoming me into your family. Rachel, Kathe, and Chloe, you make life in graduate school so much more fun and bearable. I sincerely wish all three of you, Abbie, Beka, and Sana, to be successful in your graduate career. Thank you to the three best people outside the lab, William, Mon-Ju, and June, my biggest emotional supporters during these years in graduate school. You are my people who I can safely call even in the middle of the night to talk and share my happiness as also when things get hard.

Most of all, I am forever grateful to my family, especially my parents, for all your hard work, scarification, and support for me. You have fostered my love for nature, taught me the beauty of empathy, shown me what hard work means, and believed in me for who I am. You once told me that love means scarification, and I could feel so much of your love when I look at my success in life. It is a grace for me to be your son and I wish you to always be happy.

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LIST OF ABBREVIATIONS

TERMS

ASV	Amplicon sequence variant
CAS	Chrome Azurol S
CPG	Acid–peptone–glucose medium
DAPG	2,4-diacetylphloroglucinol
ddH₂O	Double distilled water
FW	Fresh weight
GWAS	Genome-wide association study
HCN	Hydrogen cyanide
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
KB	King’s B medium
LB	Luria-Bertani medium
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PGPB	Plant growth-promoting bacteria
QTL	Quantitative Trait Loci
R2A	Reasoner's 2A medium
RIL	Recombinant Inbred Line
YPM	Yeast–peptone–mannitol medium

TOMATO GENOTYPES

BB	Bonny Best
CMII	Castlemart II
H7996	Hawaii7996
MM	Money Maker
WV	West Virginia

STATEMENT OF PUBLISHED AND COLLABORATIVE WORK

Chapter 1 was submitted as a review paper in Biochemical Society Transactions on November 2, 2022.

Chapter 2 was published as an original research paper in Journal of Experimental Botany in May 2022 on Volume 73, Issue 16, pages 5758-5772 under the DOI: 10.1093/jxb/erac228. Elizabeth French contributed to isolating the bacterial endophytes and optimizing the *in vitro* functional characterization experiments.

Chapter 3 was published as an original research paper in Phytobiomes Journal in July 2020 on Volume 4, pages 314-326 under the DOI: 10.1094/PBIOMES-02-20-0020-R. Elizabeth French is the first author of this manuscript responsible for microbial profiling and analysis of microbiome data. I performed the plant-binary interaction experiment and analyzed plant growth promotion data.

Chapter 4 is a newly developed research project.

ABSTRACT

Plant roots form an intimate relationship with a diversity of soil microorganisms. Some soil-borne microbes cause harmful diseases on crops, but others promote plant growth and enhance host resilience against stressors. Beneficial bacteria have a high potential as a strategy for sustainable agricultural management, many of which have been recognized and commercialized for improving crop growth. Unfortunately, field inoculants of beneficial bacteria often give inconsistent results due to various environmental factors hindering their beneficial properties. Improving crop production utilizing beneficial bacteria requires two approaches: 1) breeding for crops with the enhanced association for beneficial bacteria and 2) improving formulation methods for producing more potent microbial products. To contribute to these goals, we address three critical questions utilizing the tomato root microbiome as a model system. First, we asked how beneficial root-associated bacteria could be efficiently identified. We developed a strategy to select beneficial bacteria from a novel collection of 183 bacterial endophytes isolated from roots of two field-grown tomato species. The results suggest that isolates with similar traits impact plant growth at the same levels, regardless of their taxonomic classification or host origin. Next, we asked whether host genetics contribute to the root microbiome assembly and response to beneficial microbes. An assessment of the root microbiome profile and plant binary interaction experiments suggested the role of host genetics in influencing root recruitment and response to beneficial bacteria. Subsequently, we asked whether root-associated bacteria induce physiological changes in root tissues in the host. We identified two isolates from our bacterial endophyte collection that significantly promoted the growth of tomato genotype H7996 (*Solanum lycopersicum*). Plant-binary interaction experiments suggested a significant increase of cell wall lignification in the root vasculature starting 96-hour post-inoculation with beneficial bacteria. Additional studies are needed to uncover a possible correlation between the induced vasculature lignification and the growth-promoting effects of the two isolates on H7996. Altogether, our findings highlight the multi-faceted role of root-associated bacteria in promoting plant growth and support the development of crop improvement strategies in optimizing host association with soil bacteria.

CHAPTER 1. EARLY INTERACTIONS BETWEEN PLANT ROOTS AND SOIL BACTERIA: FACTORS INFLUENCING RECRUITMENT AND COLONIZATION

1.1 Abstract

Plant roots constantly encounter and form relationships with a diversity of microorganisms in the soil. Such interactions have nurtured the co-evolution process, in which soil microbes play a pivotal role in the survival and long-term fitness of the host. Although evidence of root-associated bacteria in promoting plant health is widely recognized, specific mechanisms of how the host selects for and structures its microbial community have only been recently investigated. This review discusses multiple factors used by plant roots to recruit and initiate mutualistic relationships with specific microbes from the soil microbiota. We first discuss the chemicals exuded by roots as the gateway to draw suitable bacteria closer to the root vicinity. After the microbial establishment in the rhizosphere, both the host and microbes undergo a vigorous exchange of signals to initiate colonization. We discuss the involvement of root immunity to recognize beneficial microbes and strategies of the colonizers to communicate with the host in the early stages of symbiosis. Finally, we briefly discuss the response of specific cell types during interaction with beneficial microbes and how the structural changes of these cells are crucial for maintaining symbiosis. Collectively, there is a complex multi-step process in symbiosis initiation which allows the plant to identify and exclude potential pathogens while encouraging associations with beneficial members in the soil.

1.2 Introduction

Soil harbors an incredible diversity of microbial life on earth (Torsvik and Øvreås 2002). Indeed, bacteria are among the most dominant kingdoms of all soil microorganisms and comprise 15% of the total living biomass (Bar-On, Phillips, and Milo 2018). Such bacterial communities form an intimate relationship with plant root systems (Chouhan et al. 2021; Hayat et al. 2010a; Oleńska et al. 2020; Rosenberg and Zilber-Rosenberg 2016). Root-associated bacteria typically have one of three symbiotic relationships with host roots (Naylor et al. 2017): commensalism - in which one partner derives benefit without any discernable effect on the other, mutualism - a positive interaction in which both symbiotic partners receive benefits, or pathogenicity - a negative

interaction in which the microbe cause disease to its host (Pieterse et al. 2014). The complex microbial community of soil bacteria associated with the plant root is defined as the root microbiome. Because of its close relationship with the plant, the root microbiome can directly and indirectly influence plant host health (Fitzpatrick et al. 2018; Haney et al. 2015; Hirsch 2004; Klein et al. 2013; Thoms, Liang, and Haney 2021). For example, the root microbiome can promote plant growth and development, and can reduce crop loss due to disease or abiotic stress (Berendsen et al. 2018; Orozco-Mosqueda et al. 2022; Schlatter et al. 2017). Thus, a functional microbiome in the soil is crucial for plant survival and fitness (Banerjee and van der Heijden 2022). However, the mechanisms through which the root microbiome promotes plant health are not well understood. In addition, which microbes are important and the role of the host in selecting for these microbes are not completely clear. Answering these questions is important for developing novel breeding approaches for crops with traits that can enhance symbiosis with beneficial root-associated microbes while reducing the impact of soil borne microbial pathogens (French et al. 2021).

In this review, we discuss current knowledge of the communication between beneficial bacteria and their host in the early stages of interaction. We first discuss the role of root traits in microbial recruitment. We then describe the role of host immunity in promoting the colonization of specific microbes. We also discuss strategies of beneficial bacteria to reprogram host immunity. Last, we assess how roots respond to beneficial bacteria during the initial colonization.

1.3 Host recruitment of beneficial bacteria

1.3.1 Roots exude diverse classes of chemicals into the rhizosphere to recruit suitable microbes

Plant roots exude a diversity of chemicals into the surrounding soil, turning the environment into a nutrition-rich niche suitable for nurturing symbiotic associations with soil microbes (Korenblum, Massalha, and Aharoni 2022). These chemicals have strong selective power on the soil microbiota and are often hypothesized as the first step of the host to attract suitable microbes. Root recruitment of soil bacteria involves the secretion of different classes of metabolites. These include photosynthates that are substrates for microbial growth and specialized metabolites that act as chemoattractants for specific microbes. Some metabolites have dual functions as both chemoattractants and antimicrobial compounds which exclude microbes that are incompatible with the host (Hacquard et al. 2015; Hartmann et al. 2009). Microbes that utilize and

sense metabolites from root exudates can move toward these substrates in the rhizosphere (the soil adjacent to the root surface where resources are most abundant) and initiate a relationship with the plant host. This metabolic gating mechanism is a critical factor in explaining host-specificity and the subsequent outcome of plant-microbe interaction (Rudrappa et al. 2008).

Photosynthates such as carbohydrate or sugar molecules are primarily utilized as carbon sources and may play an essential role in the rough assembly of the rhizosphere microbiota. The richness and variety of sugar types in root exudates depend on the host's developmental stage, environmental condition, and health status (Yamada et al. 2016; Zhalnina et al. 2018). Sugars are most abundantly released during the early development of plants, suggesting that the acquisition of a broader microbial population in young seedlings might be necessary before the stronger pressure from other metabolites is applied to sculpt the community.

Besides sugars, other specialized metabolites in root exudates including amino acids, organic acids, and phenolic compounds, play a regulatory role in shaping the community and may explain the genotype-specific effect of the root microbiome (Hu et al. 2018; Seitz et al. 2022). Root secretion of amino acids is highly abundant during active growth stages (Zhalnina et al. 2018). This class of metabolites appears to be essential for fostering critical symbiotic relationships. The legume *Glycyrrhiza uralensis* secretes the toxic amino acid canavanine, which imposes strong selective pressure into the rhizosphere during its vegetative stage (Cai et al. 2009). Resistance to canavanine is observed in the bacterium *Mesorhizobium tianshanense*, an important symbiotic partner, suggesting the role of co-evolution in shaping the specificity of symbiosis.

Another class of plant-specialized metabolites abundantly identified in the rhizosphere is organic acids. Organic acids likely function as a chemoattractant and as signaling molecules supporting colonization and biofilm formation of specific microbes on the root surface (Chaparro et al. 2013; Liu et al. 2014; Saleh et al. 2020). In addition, microbes can also utilize organic acids as carbon substrates, suggesting the role of organic acid in multiple metabolic activities in microbes (Cai et al. 2009; Chaparro et al. 2013; Jacoby and Kopriva 2019; Liu et al. 2014; Saleh et al. 2020; Seitz et al. 2022). Many rhizosphere bacteria isolated from *Avena barbata* possess genes annotated as transporters of diverse organic acids (Zhalnina et al. 2018). This result hints at a possible correlation between soil bacteria, organic acids in the root exudates, and their relationship with the root.

The effect of the host's exuded phenolic compounds on the root microbial community can be both positive and negative (Wang et al., 2018; Zwetsloot et al., 2018). Flavonoid, a sub-class of phenolics, is critical for rhizobium-legume symbiosis as it serves as a signaling molecule to attract suitable symbionts to the host. Another well-studied example is coumarin, an antimicrobial phenolic metabolite discovered to influence the structure of the rhizosphere microbiome in *Arabidopsis* (Stringlis, Yu, et al. 2018; Stringlis, de Jonge, and Pieterse 2019; Verbon et al. 2017; Voges et al. 2019). The exogenous application of coumarin restores the wild-type rhizosphere microbial community in mutant plants defective in the coumarin biosynthesis pathway, suggesting a direct interaction between coumarin and root-associated microbiota (Voges et al. 2019).

Host-secreted phytohormones also influence the microbial profile in roots. For example, salicylic acid (SA) and jasmonic acid (JA), two major defense hormones, significantly impact the composition of the root endophytic microbiota (bacterial community living inside the root) (Bodenhausen et al. 2014; Eichmann, Richards, and Schäfer 2021; Lebeis et al. 2015). A precursor of ethylene, 1-Aminocyclopropane-1-carboxylic acid (ACC), may be a major chemoattractant for many known beneficial inoculants, including the plant growth-promoting rhizobacteria (PGPR) *Pseudomonas putida* UW441. The effect of phytohormones on microbial assembly is challenging to examine due to its complex crosstalk with other plant metabolic pathways. Although auxin is a commonly studied hormone, its effect in a more complex microbial population can be difficult to untangle due to the extensive interconnection between auxin and other plant hormones, and its production by both plants and microbes (Boivin, Fonouni-Farde, and Frugier 2016; Kunkel and Harper 2018; Shigenaga et al. 2017; Spaepen and Vanderleyden 2011). Other root secreted metabolites that contribute to the recruitment of the root microbiome includes triterpenes, quaternary ammonium, benzoxazinoids, and camalexin (Hu et al. 2018; Jacoby and Kopriva 2019; Koprivova and Kopriva 2022; Korenblum et al. 2022; Webb et al. 2014; Zhelnina et al. 2018).

The influence of these diverse metabolites on the root microbiome suggests that the host employs multiple chemicals to attract beneficial bacteria. However, the mechanism by which root-associated bacteria perceive and respond to these metabolites remains to be investigated. Additionally, the host genes, pathways, and expression patterns that drive production of these metabolites is not always well understood in crop plants. Understanding the crosstalk between plant metabolites and soil microbe perception will enable strategies to enhance symbiotic relationships with beneficial bacteria, while limiting pathogen invasion.

1.4 Interactions between plant and beneficial bacteria during early colonization

1.4.1 Beneficial bacteria can evade host immune responses

Roots respond to microbes when host receptors recognize the presence of specific microbe-associated molecular patterns (MAMPs), structures that are well-conserved across both beneficial and pathogenic microbes (Beck et al. 2014; Lopez-Gomez et al. 2012; Millet et al. 2010; Wyrsh et al. 2015). Plants have a staggering suite of receptors, called pattern-recognition receptors (PRRs), to recognize a wide range of MAMPs (DeFalco and Zipfel 2021; Ngou, Ding, and Jones 2022; Rhodes et al. 2022). After recognizing MAMPs, plants protect themselves from potentially deadly microbes by activating pattern-triggered immunity (PTI). MAMPs from beneficial bacteria can also induce a vigorous root immune response. For example, cellular components of plant growth promoting pseudomonads can trigger immune responses in roots of both *Arabidopsis* and tobacco (van Loon et al. 2008; Millet et al. 2010; Stringlis, Proietti, et al. 2018).

How plants initiate symbiosis or immunity to some but not other members in a complex community is not well understood. Beneficial bacteria are hypothesized to have specific mechanisms to survive and proliferate through strong selective pressures from the host. For example, some non-pathogenic bacteria can conceal the presence of MAMPs during interaction with the host, thereby evading PTI. Sequence variations of the flg22 epitope, an immunogenic monomer of the bacterial MAMP flagellin, allow microbes to avoid being recognized by the host PRR receptor Flagellin Sensing 2 (FLS2) (Colaianne et al. 2021; Parys et al. 2021). Beneficial bacteria can also degrade MAMPs to avoid host detection. Homologs of the gene encoding the extracellular protease AprA are abundantly found in rhizobia and beneficial pseudomonads (Pel et al. 2014). This protease degrades flg22 monomers during interaction (Bardoel et al. 2011; Pel et al. 2014). These results suggest that root-associated bacteria can evolve ways to obscure plant immune activation before the initiation of symbiosis.

1.4.2 Beneficial bacteria can suppress host immune responses

Suppression of the host PTI response is required for pathogens to colonize plants and induce pathogenicity successfully. Similarly, many beneficial bacteria can actively suppress the host's initial immune activation, as demonstrated by plant growth promoting bacteria including *Pseudomonas simiae* WCS417, *P. putida* WCS358, and *Bacillus subtilis* FB17 (Lakshmanan et al.

2012; Millet et al. 2010; Stringlis, Proietti, et al. 2018; Yu et al. 2019). Specifically, *P. putida* WCS358 produces a subclass of organic acids in the endosphere, resulting in acidification of the surrounding environment and ultimately suppressing flg22-triggered immunity in the root (Yu et al. 2019). Beneficial bacteria also may utilize immuno-suppressive proteins secreted from the Type 3 Secretion System (T3SS) to target multiple components of PTI. The ability to synthesize T3SS has been discovered in many beneficial bacteria, including the nitrogen-fixing rhizobia and pseudomonads (Berendsen et al. 2015; Deakin and Broughton 2009; Loper et al. 2012; Stringlis, Zamioudis, et al. 2019; Teulet et al. 2022). Furthermore, metagenomic analysis on the root microbiome of different crop species indicates high enrichment of genes encoding the assembly of the T3SS (Berendsen et al. 2015; Bulgarelli et al. 2015; Ofek-Lalzar et al. 2014; Zhang et al. 2011). However, the characterization of type 3 proteins in beneficial microbes remains to be explored, and its contribution to symbiosis is primarily understood in rhizobia. The PGPR *Sinorhizobium fredii* NGR234 delivers multiple effector-like proteins called Nodulation outer proteins (Nops) through the T3SS, such as NopM and NopL, during early interaction with the host (Bartsev et al. 2004; Xin et al. 2012; Zhang et al. 2011). NopM suppresses flg22-induced reactive oxygen species (ROS) burst that is part of the early PTI response (Xin et al. 2012), while NopL is a MAP kinase substrate that interferes with the activity of defense signaling proteins and inhibits early nodule senescence (Bartsev et al. 2004; Ge et al. 2016; Zhang et al. 2011).

Some non-pathogenic bacteria have evolved ways to tolerate host immune responses rather than evade or suppress them. These microbes may actually benefit from the host defense mechanism to indirectly outcompete other microbes. *Bacillus velezensis* FZB42 root colonization promotes immune system activation in Arabidopsis. ROS produced during this activation stimulates auxin production by the bacteria. Microbially produced auxin protects the bacterium from the antimicrobial properties of ROS and facilitates bacterium proliferation on the root surface (Tzipilevich et al. 2021). Given that *B. velezensis* produces antifungal metabolites, its enhanced survival also protects the plant against fungal pathogens.

The plant host determines the outcome of its interaction with microbes by receiving multiple inputs from the bacteria or the surrounding environment. In addition to MAMPs, additional factors from beneficial bacteria can be recognized by plant receptors. The symbiotic relationship between rhizobia and its host is initiated when cognate receptors from the host perceive Nod Factors (NFs) released by the bacteria. Recognition of NFs from *Bradyrhizobium*

japonicum strongly suppresses the PTI response in soybean and Arabidopsis (Liang et al. 2013). Other microbial signals, including exopolysaccharides (EPS) and lipopolysaccharide (LPS), also play a critical role in the establishment of symbiosis (Bourassa et al. 2017; Kawaharada et al. 2015; Skorupska et al. 2006).

Some non-pathogenic bacteria can evade host immunity by strategically colonizing root cells without causing physical damage to the host (Zhou et al., 2020). Root defense responses are elicited when the host perceives dual inputs from both MAMPs and chemical signals leaking from damaged host cells (Poncini et al., 2017; Zhou et al., 2020). In contrast, activation of PTI is less intense under either cellular damage or MAMP alone, suggesting the role of host recognition utilizing multiple receptors to fine-tune root immunity (Zhou et al., 2020). This damage-gating defense allows roots to localize immunity at compromised tissues, accurately target only damaging invaders, and avoids the costly constitutive immune activation when living in the MAMP-rich environment.

Further studies are important to understand how plant roots balance the suppression of MAMP responses by beneficial bacteria with the need to increase defense responses against pathogenic microbes.

1.4.3 Cell-type specific responses may help roots recognize and establish symbiosis with specific bacteria

PRRs are transmembrane immune receptors and essential components of host immunity. PRRs are hypothesized to be well-established at locations where tissues are most vulnerable to pathogen attack (Faulkner and Robatzek 2012). The PRR FLS2 can exhibit cell- and tissue-specific expression patterns in Arabidopsis roots. FLS2 is highly expressed at infection sites, in lateral root primordia, and in distinct locations such as inner cell layers, or cells whose adjacent neighbors are enduring physical stresses (Beck et al., 2014; Zhou et al., 2020). The local MAMP perception and response depend on root cell type and tissue developmental stage (Kawa and Brady 2022). For example, young endodermal cells in Arabidopsis are hyperreactive to MAMPs while fully differentiated endodermal cells function as an important checkpoint, contributing to the restriction of MAMP penetration into the vasculature (Zhou et al., 2020). PTI responses may be cell autonomous, demonstrating a tight regulation from the host on immunity.

Changes in cell wall composition and integrity in response to beneficial bacteria can be cell-type specific. Non-pathogenic bacteria have been reported to compromise the barrier of specialized root cells and instead of causing detrimental effects to the host, they increase host fitness under unfavorable conditions. The endodermis, consisting of the lignified Casparian strip and the suberized cell wall, is a physical barrier regulating access of nutrients and microbes into the vasculature. Some beneficial bacteria can actively modify the endodermis cell wall composition, which increases mineral absorption in roots and enhances host resilience under environmental stresses (Salas-González et al. 2021). Pathogenic bacteria can also cause cell wall changes in specific cell types and tissues. For example, the cell wall of the xylem vessel is reinforced with lignin following pathogen attack, which contributes to impeding pathogen colonization (Kashyap et al. 2022; Novo et al. 2017). This observation suggests the ability of individual root cells to localize the invading microbes and avoid microbial progression into deeper tissues.

Additional work is needed to understand the mechanisms that beneficial bacteria use to impact different root cell types and tissues, and how these changes lead to increased plant health and fitness. Such knowledge could be used to deploy specific mixes of microbial inoculants that promote plant health through different tissue-specific mechanisms. These types of inoculants could provide enhanced plant growth promotion and protection to a broad range of environmental stresses.

1.5 Conclusions

Although soil is a determining factor for shaping the microbial composition in the root, the host also significantly impacts recruitment, selection, and functioning of the root microbiome. Integrated strategies investigating how roots communicate with soil bacteria and how specific interactions become mutualisms will open new horizons for developing crops with root systems optimized for association with beneficial microbes while mitigating the detrimental effect from environmental stress.

1.6 Perspectives

- Plant host selection of root-associated beneficial microbial communities has great potential to sustainably increase crop production and protect crops against environmental stress.
- Host recruitment patterns suggest plants attract microbes with specific functions, regardless of their taxonomic classification. Members of the root microbiome can evade, suppress or tolerate host immunity to promote colonization. Root-associated microbes colonize plants and alter plant growth and responses to stress in part through impacts on root cell types and tissues.
- Further studies aimed at understanding the molecular strategies plants use to recruit bacteria and enable colonization, while preventing pathogen infection, are needed. What host genes are required for recruitment? How do bacteria perceive host metabolites and use the metabolites for their benefit? How common is it for beneficial bacteria to impact specific root tissues? Which root tissues are impacted, and how do these changes enhance plant health?

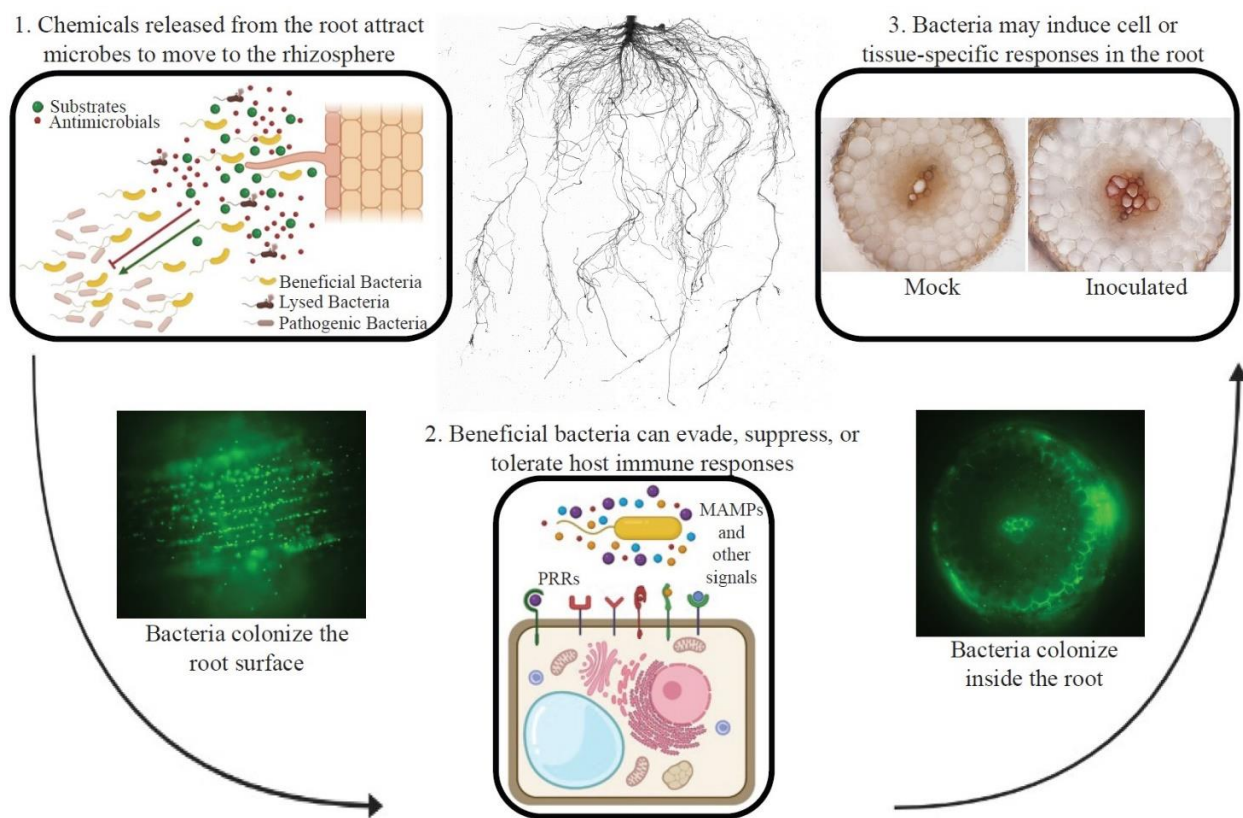


Figure 1.1. Three steps by which plant roots recruit and communicate with beneficial bacterial endophytes during the early stages of interaction.

1. Roots secrete various chemicals into the surrounding soil to attract beneficial bacteria while excluding incompatible microbes. These chemicals function as substrates for microbial metabolism and/or as antimicrobials to select for specific microbes. Once present in the root rhizosphere, microbes must attach to the root and colonize it. Image shows GFP-tagged *Pseudomonas* colonizing the tomato root surface. 2. Beneficial bacteria have multiple strategies to evade the host immune system, such as hiding or degrading MAMPs, preventing MAMP recognition, or secreting proteins into the host cell with the type 3 secretion system. Image shows GFP-tagged *Pseudomonas* colonizing a tomato root. 3. Successful colonizers may influence the host phenotype in a cell-type specific manner, providing beneficial properties of the microbes to their host. Image generated in part with BioRender.

1.7 References

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CHAPTER 2. *IN VITRO* FUNCTIONAL CHARACTERIZATION PREDICTS THE IMPACT OF BACTERIAL ROOT ENDOPHYTES ON PLANT GROWTH

2.1 Abstract

Utilizing beneficial microbes for crop improvement is one strategy to achieve sustainable agriculture. However, identifying microbial isolates that promote crop growth is challenging, in part because using bacterial taxonomy to predict an isolate's effect on plant growth may not be reliable. The overall aim of this work was to determine whether *in vitro* functional traits of bacteria were predictive of their *in planta* impact. We isolated 183 bacterial endophytes from field-grown roots of two tomato species, *Solanum lycopersicum* and *S. pimpinellifolium*. Sixty isolates were screened for six *in vitro* functional traits: auxin production, siderophore production, phosphate solubilization, antagonism to a soilborne pathogen, and the presence of two antimicrobial metabolite synthesis genes. Hierarchical clustering of the isolates based on the *in vitro* functional traits identified several groups of isolates sharing similar traits. We called these groups 'functional groups'. To understand how *in vitro* functional traits of bacteria relate to their impact on plants, we inoculated three isolates from each of the functional groups on tomato seedlings. Isolates within the same functional group promoted plant growth at similar levels, regardless of their host origin or taxonomy. Together, our results demonstrate the importance of examining root endophyte functions for improving crop production.

2.2 Introduction

Plant roots form an intimate relationship with a diversity of bacteria in the soil, the largest reservoir for microbial life on earth (Torsvik and Øvreås, 2002). While some root-associated bacteria are pathogens, others are capable of promoting plant growth and alleviating the negative effects from both biotic and abiotic stressors (Klein et al., 2013; Naylor et al., 2017; Fitzpatrick et al., 2018; Li et al., 2021). Because of their beneficial contributions to plants, identification of beneficial bacteria provides new opportunities for crop improvement. However, selection of specific bacterial isolates that lead to plant growth promotion or disease suppression traits in greenhouse or field environments is challenging. Here, we asked whether we could efficiently

predict the impact of non-pathogenic bacteria on plant growth in a greenhouse setting. We hypothesized that screening for multiple functional traits *in vitro* is a reliable tool to predict the impact of root associated bacteria on promoting plant growth in the greenhouse.

The constant interaction between beneficial soil bacteria and plant hosts fosters an evolutionary symbiotic partnership (Rosenberg and Zilber-Rosenberg., 2016). Root-associated bacteria often have a profound impact on plant growth and development (Hayat et al., 2010; Oleńska et al., 2020; Chouhan et al., 2021). Beneficial bacteria have a wide range of biological mechanisms to stimulate plant growth and enhance host resilience against stress. These include improving plant nutrient uptake through the production of iron-chelating siderophores (Trapet et al., 2016) and the secretion of enzymes that mobilize inorganic phosphate to a plant-available form (Alori et al., 2017; Bargaz et al., 2021), promoting plant growth through the production of the hormone auxin indole-3-acetic acid (IAA) (Tzipilevich et al., 2021), and inhibiting pathogenic plant microbes through the secretion of antimicrobial secondary metabolites (Compant et al., 2005) like 2,4-diacetylphloroglucinol (DAPG), and hydrogen cyanide (HCN) (Couillerot et al., 2009; Paulin et al., 2017).

Root-associated bacteria have great potential for use in sustainably managing agricultural systems (French et al., 2021). Over decades, many bacterial isolates have been recognized and commercialized for improving crop growth (García-Fraile et al., 2015). These biofertilizers are formulated either from a single strain (Azotobacterin ® by JSC “Industrial Innovations”) or from a combination of microbial consortia (Life ® by Biomax). Unfortunately, the application of commercial inoculants often gives inconsistent results in the field, even when they contain beneficial bacteria that are known to have a tight association with the host (French et al., 2021). The introduced inoculants often cannot thrive in the field environment in which they are applied, either because they are outcompeted by native soil microorganisms or they are incompatible with soil edaphic factors.

The ability of a microbe to positively impact plant growth and development depends on multiple factors, including microbial taxa, host genotype, and the surrounding environmental conditions. Predicting which root-associated bacteria promote plant growth in a given environment is therefore extremely difficult. Functional traits of microbes can be useful to predict the outcome of plant-microbial interactions (Kia et al., 2017; Giauque et al., 2019) because they can provide a glimpse of a given microbe’s ability to alter host physiological processes. Very broadly, microbial

functional traits are conserved with phylogeny, but this relationship depends on several factors, including microbial habitat, depth of taxonomic rank considered, and trait complexity (Philippot et al., 2010; Martiny et al., 2013, 2015; Goberna and Verdú, 2016; Morrissey et al., 2019). Determining the relationship between functional trait and phylogeny in the outcome of root-bacteria interactions would be useful for developing microbial inoculants of single isolates or consortia for use in sustainable agriculture.

Here, we examine whether functional traits and taxa of bacterial isolates obtained from inside the root (root endophytes) of healthy tomato plants predict the impact of these isolates on plant growth. We reasoned that the high selection pressure and narrow environmental conditions of the root endosphere may enrich these microbes with traits that promote plant growth. We assessed functional traits that likely reflect microbial pathways that impact host plants, including hormone and antimicrobial production (Naik et al., 2008). The objectives of this study were to 1) determine whether the characterization of multiple *in vitro* functional traits of root-endophytic bacteria are informative regarding an isolate's impact on plant growth, 2) determine whether isolates with similar *in vitro* functional traits similarly impact plant growth, and 3) determine whether root endophytic bacteria related at high taxonomic resolution similarly impact growth.

We isolated bacterial root endophytes from field-grown roots of two tomato genotypes, a domesticated tomato species *Solanum lycopersicum* Hawaii7996 (H7996) and its wild cousin *S. pimpinellifolium* West Virginia (WV). We phylogenetically identified each isolate through sequencing of the 16S rRNA gene and characterized isolates for six functional traits. We classified isolates into groups based on their *in vitro* functions and tested three members of each group for plant growth promoting ability using plant-binary interaction experiments. Our results suggest that phenotyping multiple *in vitro* functional traits of culturable root endophytic bacteria can predict the isolates' impact in planta, and that the functional traits of bacterial root endophytes are more important for plant growth promotion than taxonomic status, at least at high taxonomic resolution.

2.3 Materials and Methods

2.3.1 Isolation and identification of root bacterial endophytes in tomato

Endophytes were isolated from roots of two tomato genotypes, *S. lycopersicum* 'Hawaii7996' (H7996) and *S. pimpinellifolium* accession West Virginia700 (WV). Plants were grown at

Throckmorton Purdue Agriculture Center (40.2° N, 86.9° W). Seedlings were planted in May 2017 and three plants of each genotype were harvested in August 2017. After harvest, roots were carefully washed to remove debris. Representative portions of each root area (primary, secondary and tertiary roots) were excised from each plant. Cleaned root portions were surface sterilized by soaking in 5% bleach with shaking for three minutes and rinsed five times with sterile ddH₂O. The last sterile water rinse was plated on Luria-Bertani (LB) media and no bacterial growth was observed. Tissue was macerated and homogenized with sterile 1x phosphate-buffered saline (PBS) (pH=7.0). 100 µL homogenized liquid was plated onto three different media types: Luria-Bertani (LB) for recovery of copiotrophs, R2A for recovery of oligotrophs, and King's B medium for selection of the pseudomonads. In total, 183 bacterial endophytes were isolated (Appendix Figure A2.1).

The culturable endophytes were named after the host in which they were originally isolated in numerical order of isolation. For example, isolate WV-44 was the 44th isolate recovered from roots of WV; isolate HA-88 was the 88th isolate recovered from H7996. The endophytes were subsequently identified by Sanger sequencing of the 16S rRNA subunit using primers commonly used for bacterial 16S rRNA sequencing: 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1392R 5'-ACG GGC GGT GTG TAC A-3'. Sequence results were then aligned to the NCBI databases for taxonomic identification.

To determine the taxonomic relationship between isolates, the 16S rRNA sequences were analyzed with T-Coffee multiple sequence alignment program (Madeira et al., 2019) using default parameters for DNA. Based on sequence similarities, the isolates were classified into one of the three categories: H7996 exclusive, which are root endophytes isolated from only H7996; WV exclusive, which are root endophytes isolated from only WV; and high frequency, which are isolates with sequence found from both hosts.

2.3.2 *In-vitro* characterization of functional traits of bacterial endophytic isolates

PCR-Based Screening for Biocontrol Potential

All isolates were PCR-screened for 2,4-Diacetylphloroglucinol (DAPG) and Hydrogen cyanide (HCN) biosynthesis genes *phlD*, and *hcnAB* respectively, using the following primers:

phlD-F: 5'-ACC CAC CGC AGC ATC GTT TAT GAG C-3'; *phlD*-R: 5'-CCG CCG GTA TGG AAG ATG AAA AAG TC-3'; *hcnAB*-F: 5'-TGC GGC ATG GGC GTG TGC CAT TGC TGC CTG G-3'; *hcnAB*-R: 5'-CCG CTC TTG ATC TGC AAT TGC AGG CC-3'.

In-vitro antagonism against R. solanacearum

Antagonistic potential against the soilborne pathogen *R. solanacearum* strain K60 was screened utilizing the dual-culture method. 200µL of 10⁸ CFU/mL *Ralstonia* was evenly streaked on Casamino Acid-Peptone-Glucose (CPG) with 0.2% triphenyltetrazolium chloride (TZC) plates. The streaked plates were incubated at 28°C for four hours, after which 1 cm diameter wells were created on the agar surface. 50µL of 10⁸ CFU/mL endophyte inoculum or LB as a control was subsequently placed into the wells and incubated at 28°C. After three days, the area of inhibition was quantified using ImageJ software (Schneider et al., 2012). Each isolate was tested three times in each of three independent experiments.

Production of Indole Acetic Acid (IAA)

Microbial IAA was quantified as described by (Gordon and Weber 1951). Briefly, the endophyte inoculums were liquid-cultured in 4mL Yeast-Peptone-Manitol liquid medium (YPM) supplemented with 0.1% Tryptophan for four days. 1mL of inoculum was centrifuged, supernatant removed, and the precipitate was dried at 50°C for 2 days. Six controls at 0, 5, 10, 20, 50, and 100µg/mL were prepared by mixing IAA with 0.1% Tryptophan-supplemented YPM liquid medium. 1mL supernatant was mixed with 2mL of Salkowski Reagent (2mL 0.5M FeCl₃, 49mL sterile H₂O, and 49mL 70% perchloric acid) and incubated for 25 minutes and the six standards. After incubating for 25 minutes, colorimetric quantification was determined at OD530. IAA concentration per gram of dry cell (µg/g) was calculated using the trendline equation plotted from the optical density of the six standards. Each isolate was tested three times in each of three independent experiments.

Siderophore Production

Siderophore production was screened by growing the isolates on Chrome Azurol S (CAS)-LB plates as described in (Louden et al., 2011). Briefly, CAS-LB agar was prepared by mixing the CAS-FeCl₃ dyeing agent into LB agar (pH 6.8). CAS-FeCl₃ was prepared from three solutions:

0.06g CAS in 50mL ddH₂O, 0.0027g FeCl₃.6H₂O in 10mL ddH₂O, and 0.073g Hexadecyltrimethylammonium Bromide (HDTMA) in 40mL in 10mL ddH₂O. After preparation, 9mL of solution two was mixed with 50mL of solution one, before adding 50mL of solution three. The dying agent was autoclaved separately before adding to 900μL LB agar medium to create CAS-LB plates. Wells with 1 cm diameter were subsequently created on the agar surface. 50μL of 10⁸ cfu/mL endophyte inoculum or LB as a control was placed into the wells. After 60 minutes, plates were incubated at 28°C. After 72hr, the surface area of the halo zone was quantified using the ImageJ software (Schneider et al., 2012). Each isolate was tested three times in each of three independent experiments.

Solubilization of Mineral Phosphate

The ability to solubilize inorganic phosphate was screened by growing isolates on Pikovskayas agar plates, a medium enriched with Ca₃(PO₄)₂. The protocol for preparation of Pikovskayas agar was described in (WVB Sundara Rao and Sinha, MK, 1963). Before the experiment, wells with the diameter of 1cm were created on the agar surface. 50μL of 10⁸ cfu/mL endophyte inoculum, or LB as a control, was carefully pipetted into the wells. After 60 minutes, plates were incubated at 28°C. Surface area of the halo zone was quantified using the ImageJ software (Schneider et al., 2012). Each isolate was tested three times in each of three independent experiments.

Data Analysis and Visualization

Each of the 64 isolates screened for *in vitro* functional traits had three data points from three replications for each of the four traits which were quantitatively measured: antagonism, auxin production, siderophore production, and phosphorus solubilization. The average data of the three experimental replications for each of these traits was calculated for every isolate. To standardize all features, a Z-score was calculated for each of these traits on individual isolates with the following formula:

$$Z \text{ score for specific trait} = \frac{\text{average from three reps of that trait} - \text{average of all four traits}}{\text{Standard deviation of all four traits}}$$

Analyses were performed and visualized in R (v 1.4.0)(R Core Team). Heatmaps were constructed with the package pheatmap (Kolde, 2012) by performing hierarchical clustering utilizing Euclidean distance on the Z-scores for each isolate for the traits antagonism, auxin production, siderophore production, and phosphorus solubilization. In addition to quantitative data, qualitative data of the isolates included presence of *PhlD* and *hcnAB* genes, host origin and taxonomic status. These data were not clustered, but were added as additional columns to the heatmap after clustering. To determine correlation between traits, different *in vitro* functions were compared utilizing the non-parametric Kruskal-Wallis Rank Sum test using the function `kruskal.test()` in the Stats package.

2.3.3 Characterization of effects of bacterial isolates on phenotype of tomato seedlings

Plant binary interaction experiment

Plant binary interaction experiments were performed on seedlings of H7996 genotype with each of the 21 selected isolates. The experiment was conducted in a light- and temperature-controlled green house (light cycle: 16-hour of light and 8-hour of darkness, temperature setting: 24°C - 29°C). Seeds were surface sterilized with 20% bleach with gentle shaking for 10 min, rinsed five times in sterile ddH₂O and incubated in 4°C for 24 hours before germination. Seeds were sown the next day in autoclaved Metro Mix propagation mix (Sun Gro® Horticulture, USA). At seven days old, 10 seedlings were inoculated with each of the 21 selected bacteria with the concentration of 10⁷ CFU/g soil and another 10 were mock inoculated with 1X PBS (pH = 7.4) as controls. Treated plants were organized in a random complete block design for a total of ten blocks. Each block included one plant of each treatment, including both bacteria and mock-treated samples. All plants were fertilized with Peter's Excel 15-5-15 Cal Mag at 1.6 g/plant at two days after inoculation. Plants in all blocks were harvested at 21 days old to determine their fresh weight of shoot and root at the time of harvest. After root harvest, the soil sample was incubated with 1X PBS (pH = 7.4) and the wash solution was dilution plated on appropriate media (LB, KB, or R2A, depending on the media from which the strain was originally isolated). All recovered isolates were identified by morphology and colony characteristics on the media.

Data Analysis of plant responses to bacterial isolates

All statistical analyses were performed in R (v 1.4.0) (R Core Team). Plant growth promotion effect was measured by response rate, which was calculated by the change in fresh weight of both shoot and root of bacteria-inoculated plants compared to the mock-treated control within the same block. In total, ten response rates per bacteria were obtained from ten blocks. We used linear models (LMs) to examine plant responses. We compared the performance of three isolates in the same functional group and also performances between the bacterial functional groups. LMs were run using the `lm()` function in Stats package. Two-way analysis of variance (ANOVA) and Tukey post-hoc test was performed using the `aov()` and `TukeyHSD()` functions in the Stats package.

To test for correlation between phylogenetic conservation of the 21 tested isolates and their effect on the plant host, Blomberg's K and Pagel's λ were computed for plant response rates of both shoot and root with the 16S rRNA sequences using the `phylosig()` function in the Phytools package (Revell, 2012). The two most abundant bacterial families among the 21 isolates, *Pseudomonadaceae* and *Bacillaceae*, were further compared with one another for their ability to promote plant growth using the non-parametric Mann-Whitney U test.

To understand how plant responses were influenced by individual quantitative traits, we used ordinary linear regression. The dependent variables were the change in fresh shoot or root weight calculated as plant responses to the bacterial isolates. The data of four quantitative traits for the 21 selected isolates obtained from *in vitro* characterization experiment and the functional groups classified from the heatmap were included as independent variables. Linear correlations between the isolates' *in vitro* traits and plant response were determined with Pearson's correlation coefficient (r). Regressions were run in R (v 1.4.0) with model selection based on the Akaike Information criteria corrected for small sample sizes (AICc) (Akaike, 1974) using the `aictab()` function in `AICcmodavg` package (Mazerolle, 2017). Variables that appeared in top models from AICc were identified as factors that explained the most variation in plant responses.

2.4 Results

2.4.1 Generation of a culturable collection of tomato root endophytic bacteria

To investigate the relationship between *in vitro* functions and plant growth promotion, we first isolated a collection of 183 bacterial root endophyte isolates. Bacteria were isolated from the roots of six healthy field-grown tomatoes: three Hawaii 7996 (H7996, a domesticated cultivar; *Solanum lycopersicum*) and three West Virginia 700 (WV; *S. pimpinellifolium*, a wild species closely related to *S. lycopersicum*). H7996 and WV are two genotypes widely used to study resistance mechanisms against the soilborne bacterial pathogen *Ralstonia solanacearum* (Wang et al., 2000; French et al., 2018). In this collection of 183 isolates, the bacteria were differentiated based on differences in morphological appearance, media type on which they could be cultured, and host origin. Our collection comprised 86 isolates from H7996 and 97 from WV (Figure 2.1A).

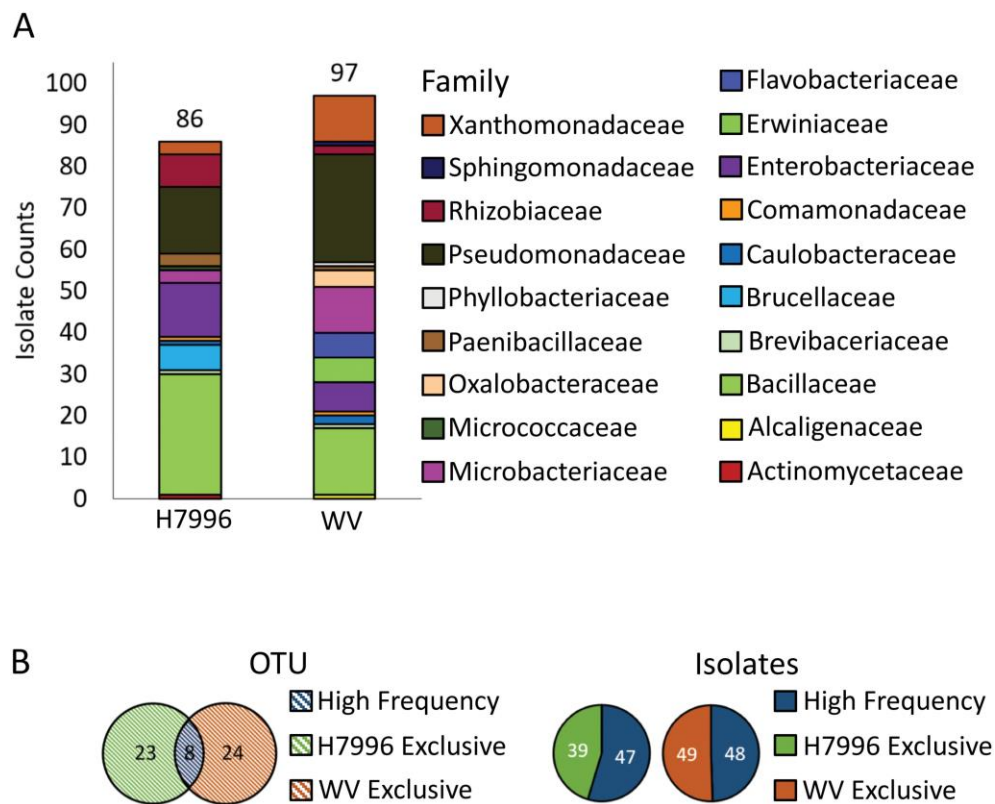


Figure 2.1. OTUs. (A) Stacked barplot of isolate counts at the family level of the endophytes in the collection and their host origin. (B) Pie charts show OTU classification of the isolates of each of the hosts. Isolates share the same OTU when the sequences of their 16S rRNA gene are $\geq 99\%$ identical. H7996, *S. lycopersicum* cv. Hawaii7996; WV, *S. pimpinellifolium* accession West Virginia700.

We first identified all isolates using Sanger sequencing of the 16S rRNA gene with the universal 27F primer. Based on the 16S rRNA sequencing data, the isolates were classified as the same OTU if they shared 99% or greater identical sequence. We categorized the 183 isolates into a total of 55 Operational Taxonomic Units (OTUs) (Appendix Table A2.1). From 55 OTUs, eight OTUs were present in both hosts, while 23 were exclusively present in H7996, and 24 were present exclusively in WV (Figure 2.1B). We called the eight OTUs present in both hosts as ‘high-frequency OTUs’. Forty-seven isolates from H7996 and 48 isolates from WV belonged to one of these eight high-frequency OTUs (Figure 2.1B). OTU that were found in only one host were called ‘host-exclusive OTUs’. Thirty-nine isolates belonged to one of the 23 H7996-exclusive OTUs while 49 isolates were found in one of the 24 WV-exclusive OTUs (Figure 2.1B).

The taxonomic structure of our 183 isolates revealed four phyla (Appendix Table A2.1). *Proteobacteria* contributed to the majority of the endophyte collection (60%) and included *Alphaproteobacteria* (21 isolates), *Betaproteobacteria* (7 isolates), and *Gammaproteobacteria* (82 isolates). Approximately 27% of our isolates were from the phylum *Firmicutes* (49 isolates), 10% (18 isolates) were *Actinobacteria*, and approximately 3% were *Bacteroidetes* (6 isolates). Of the four phyla, taxonomic classification revealed 19 bacterial families, two of which comprised 47.5% of our collection: *Bacillaceae* of the phylum *Firmicutes* (45 isolates), and *Pseudomonadaceae* of the phylum *Proteobacteria* (42 isolates) (Appendix Table A2.1). The endophytes isolated from WV were slightly more diverse, with 16 families compared to 13 families in H7996 (Figure 2.1).

The taxonomic profile of this endophyte collection is relatively consistent with the MiSeq Illumina 16S rRNA amplicon sequencing of tomato root endophytes from (French, Tran, and Iyer-Pascuzzi 2020). They observed that *Proteobacteria* composed a significant majority of tomato root endophytic microbiota (~70%,) followed by *Actinobacteria* (15.0%), *Firmicutes* (6.8%), and *Bacteroidetes* (2.6%). Here, we only identified approximately 10% of members in our collection as *Actinobacteria* (18 isolates), which might be due to the low culturability rate of this specific phylum using common selection media (Messaoudi, Wink, and Bendahou 2020).

2.4.2 Tomato root endophytes vary in functional traits

To examine the *in vitro* functional traits of the root endosphere bacteria, we selected a subset of 60 isolates representing the genetic diversity of the collection. These isolates included at least one member of every OTU identified. To represent the eight high-frequency OTUs, we first

selected one isolate from each high-frequency OTU from each host. For one high-frequency OTU, we selected two isolates from the WV host. This resulted in a total of 17 high-frequency isolates (eight from H7996 and nine from WV) (Appendix Figure A2.2). We next selected one isolate representing each of the other 47 host exclusive OTUs. We could not recover four host-exclusive OTUs from our frozen stock collection. Thus, 43 isolates representing 43 host-exclusive OTUs were included, 22 from H7996 and 21 from WV (Appendix Figure A2.2, Appendix Table A2.1; WV-44 and WV-162). In total, we selected 60 isolates, representing 51 OTUs (eight high-frequency OTUs, 22 H7996-exclusive OTUs and 21 WV-exclusive OTUs), for an *in vitro* functional screening experiment. As positive controls, we included four additional well-characterized plant growth promoting strains of *Pseudomonas* spp: *P. protegens* CHA0 and Pf-5, and *P. fluorescens* Pf01 and SBW25 (Jousset et al. 2014; Paulsen et al. 2005; Silby et al. 2009).

We screened the 60 isolates and four positive controls for six functions known to be features of plant growth promoting bacteria (Souza, Ambrosini, and Passaglia 2015), including three traits likely involved in plant growth promotion and three in competition (Appendix Figure A2.1B). For the former, we quantified the ability of each isolate to produce auxin, siderophores, and solubilize inorganic phosphate (see Materials and Methods for details). The competition traits examined included the presence of *phlD* and *hcnAB* in the genome. *phlD* and *hcnAB* are responsible for biosynthesis of two antagonistic secondary metabolites, 2,4-Diacetylphloroglucinol (DAPG) and Hydrogen Cyanide (HCN), respectively (Zhou et al., 2012). The last competition trait we quantified was antagonism against the soilborne bacterial pathogen *Ralstonia solanacearum*. We chose *R. solanacearum* in the antagonism screening because the selected bacteria were originally isolated from roots of two tomato species often used to study resistant mechanisms against this pathogen (French et al. 2018; Wang et al. 2000). We tested each of the 64 isolates three times in each of three independent experiments for each of the six *in vitro* functional traits.

From a total of 64 isolates, more than half (35 or 58.33%), tested positive for production of the phytohormone auxin IAA (Figure 2.2). Twenty-two isolates (38.33%) produced siderophores, and 21 (36.67%) showed antagonism against the pathogen *R. solanacearum*. Only nine isolates (15%) could solubilize inorganic phosphate. PCR screening revealed 21 isolates (35%) that could potentially produce DAPG, but only five (8.33%) tested positive for the HCN biosynthesis gene (Figure 2.2). Some isolates tested positive for more than one functional trait.

For example, all four well-characterized plant growth promoting bacteria produced siderophores and were antagonistic to *R. solanacearum* (Appendix Table A2.2).

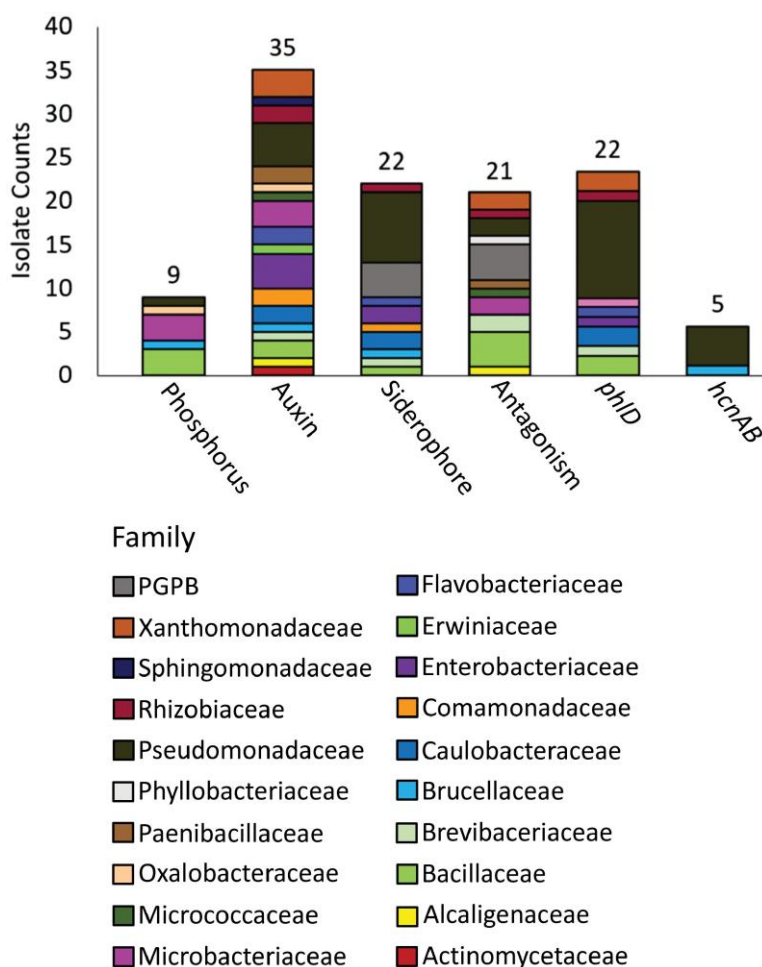


Figure 2.2. Summary of functional traits produced by the 64 tested isolates. Stacked barplot of isolate counts at family level that tested positive for the six *in vitro* functional traits listed on the x-axis.

Isolates with each functional trait were identified from both tomato hosts regardless of whether the endophyte was a high-frequency or host exclusive OTU (Figure 2.3). We did not observe any patterns between a specific taxonomic family and its functional traits (Figure 2.2, 2.3, Appendix Figure A2.3). For example, three high-frequency isolates that share the same OTU classification, WV-44, WV-162, and HA-88 (Appendix Table A2.1), exhibited different *in vitro* functional traits (Appendix Figure A2.3). Although all could produce auxin, only isolate WV-162 could also secrete siderophores. PCR screening revealed that the genomes of WV-44 and HA-88 harbored the HCN biosynthesis gene *hcnAB*. However, the presence of the DAPG biosynthesis gene *phlD* was only found in HA-88 and WV-162.

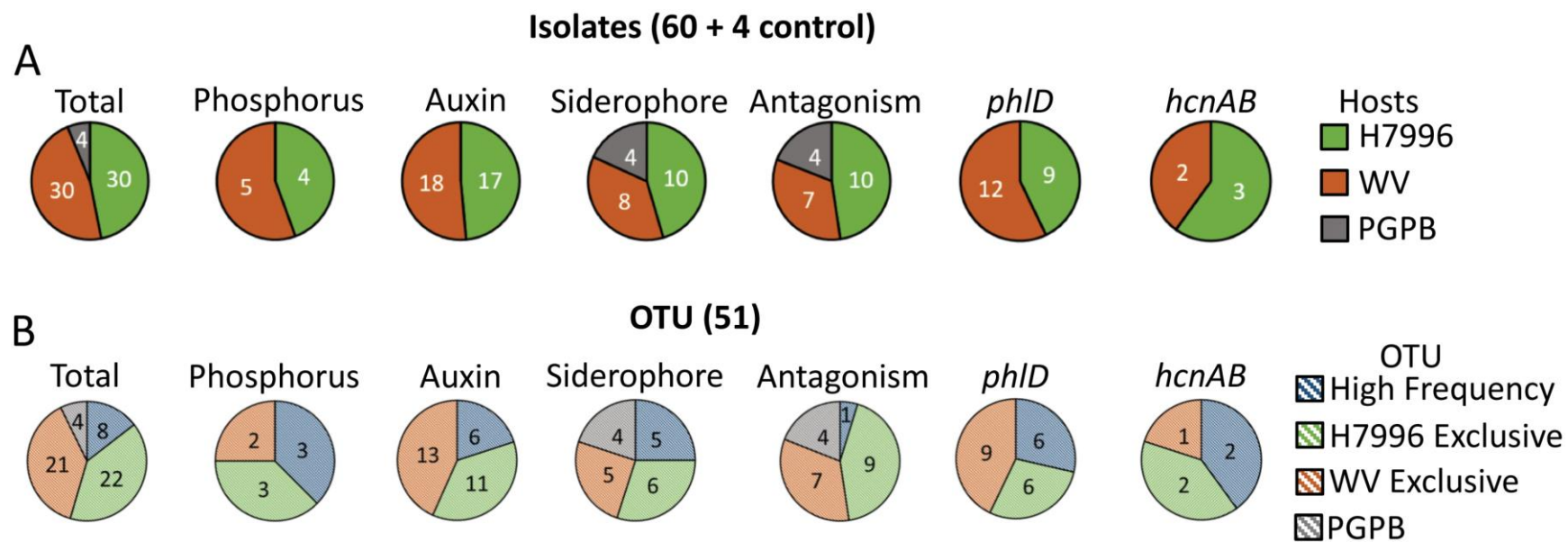


Figure 2.3. Pie charts showing the host origin for the endophytes as isolates (A) and OTUs (B) for each functional trait. H7996, *S. lycopersicum* cv. Hawaii7996; WV, *S. pimpinellifolium* accession West Virginia700.

2.4.3 Endophytes clustered into multiple groups based on four *in vitro* functional traits

To gain further insight into the relationships between endophytes and their *in vitro* functional traits, we converted trait values for auxin, siderophore production, phosphate solubilization, and antagonism to Z-scores and hierarchically clustered traits from all 64 isolates utilizing these scores (Figure 2.4). Hierarchical clustering revealed four major groups of endophytes, each of which exhibited one strong trait (defined as a Z-score above 0.5): Phosphorus solubilizers, Auxin producers, Siderophore producers, and a group of isolates that could inhibit *in vitro* growth of *R. solanacearum* (Figure 2.4). We called these groups ‘functional groups’. We also identified four other, smaller, functional groups of isolates that had a combination of two traits (Z-score > 0.5 for two groups). These included Phosphorus-Auxin, Auxin-Siderophore, Auxin-Antagonism, and Siderophore-Antagonism.

The four plant-growth-promoting *Pseudomonas* spp which we used as controls were in a functional group with a combination of two functions, Siderophore-Antagonism (Figure 2.4). The Phosphorus-Auxin group was comprised of only two isolates, making it the smallest group. The Auxin producing group had the highest number of isolates (19) and is also the most phylogenetically diverse group with 12 families (Appendix Figure A2.4). Although isolates in the bacterial family *Pseudomonadaceae* were a majority of those we screened (42 isolates), only two members of this family exhibited antagonistic activity against *R. solanacearum* (Figure 2.2). These two isolates were identified as HA-141 and WV-182, and were classified in two different two-function groups, Siderophore-Antagonism and Auxin-Antagonism, respectively (Appendix Figure A2.3).

We observed several relationships among the *in vitro* functional traits. For example, the group of isolates producing high levels of auxin did not inhibit the *in vitro* growth of the soilborne pathogen *R. solanacearum*, while those that were highly antagonistic to *R. solanacearum* did not produce auxin (Figure 2.4). In addition, 21 of the 60 characterized isolates tested positive for the presence of the *phlD* gene, which encodes an antimicrobial DAPG enzyme. These *phlD*-positive isolates tended to be strong siderophore producers (Figure 2.5). Neither the *phlD* nor *hcnAB* genes were correlated with the presence of the antagonism trait (Appendix Figure A2.5).

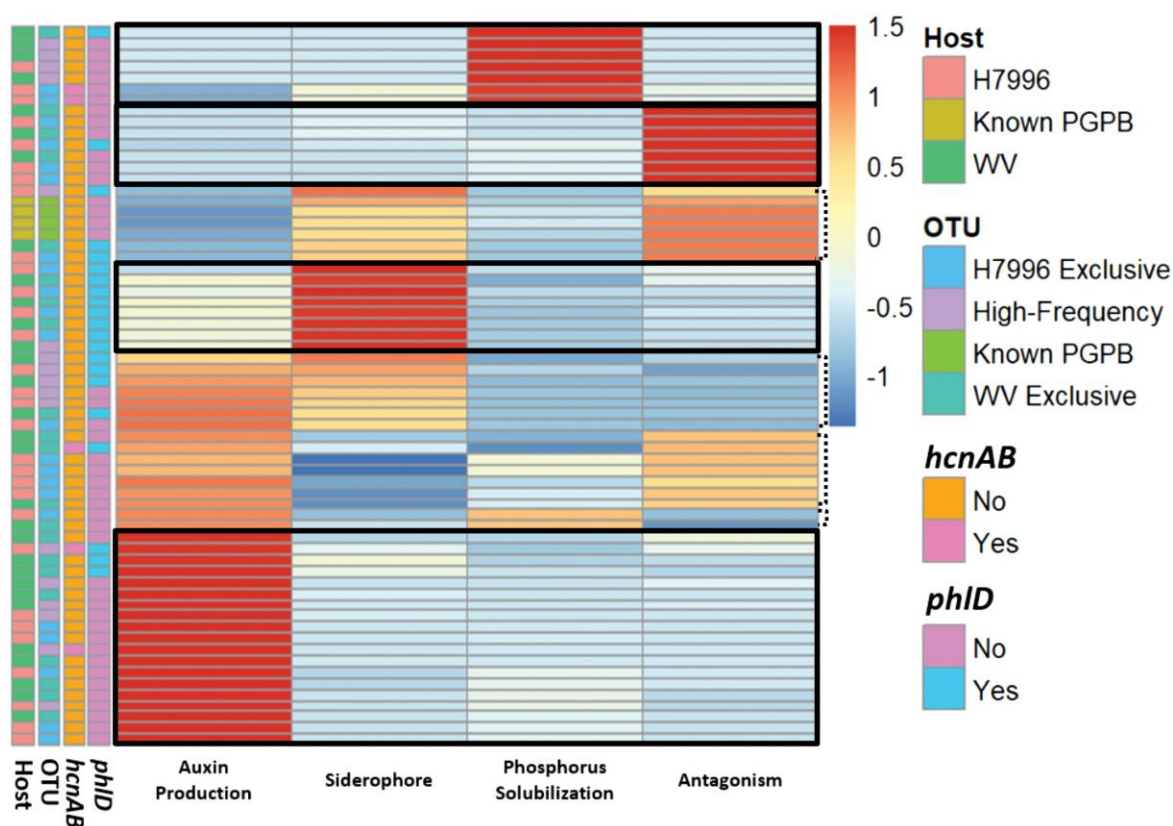


Figure 2.4. Hierarchical clustering of four functional traits of the 64 tested isolates. Each row of the heatmap represents one isolate and each column represents a functional trait. Clustering was only performed for the quantitative data using the average Z-score for each trait for each isolate across three experimental replicates. Qualitative data were added to the figure after clustering. Colors in each block of quantitative traits show the Z-score value. Black boxes indicate functional groups consisting of one function; dotted brackets indicate groups with two functions. H7996, *S. lycopersicum* cv. Hawaii7996; WV, *S. pimpinellifolium* accession West Virginia 700.

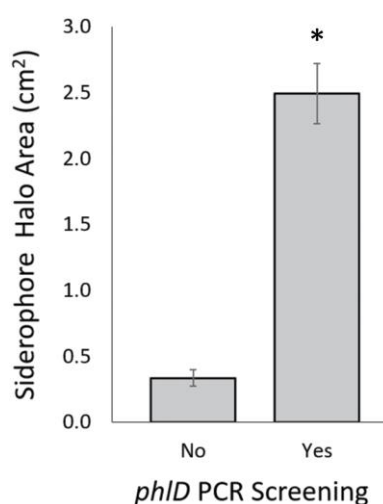


Figure 2.5. Barplot showing the relationship between siderophore-producing ability and the presence of the *phlD* gene. The 21 isolates with *phlD* have significantly increased siderophore production compared with the 39 isolates that do not carry *phlD*. The asterisk indicates significantly different at $P < 0.05$ with Kruskal–Wallis test.

2.4.4 Bacterial endophytes in the same *in vitro* functional group have similar impacts on plant growth

We hypothesized that root endophytes with similar *in vitro* functional traits have comparable impacts on plant growth. To test this, we performed plant-bacteria binary interaction experiments on a subset of isolates from our collection of 60 *in vitro* functionally characterized endophytes. We first selected three isolates from seven of the eight functional groups described above, for a total of 21 isolates (Table 2.1 and Appendix Figure A2.3). Since there were too few isolates classified in the Phosphorus-Auxin group, the isolates in this group were not included for the plant-binary interaction experiment. *P. protegens* CHA0 (a member of the Siderophore-Antagonism group) was included as a positive control for the plant growth experiment because this isolate has been demonstrated to enhance growth of tomato seedlings (Esfahani et al. 2016). Thus, the 21 selected isolates included 20 culturable endophytes, and one positive control (Table 2.1).

Of the 20 endophytes, ten of each were isolated from H7996 and WV. Based on the OTU classification, 16 of the 20 selected endophytes were phylogenetically distinct from one another, with the exception of two pairs of isolates, WV-94 and HA-28 of the OTU 5 and WV-44 and WV-162 of the OTU 11 (Table 2.1, Appendix Figure A2.3). Notably, although two members of each pair shared the same OTU, they were classified in different *in vitro* functional groups. In each pair, one belonged to the Auxin group and another belonged to Auxin-Siderophore group (Table 2.1).

We tested the plant-growth promoting abilities of each of the 21 isolates using seven-day-old H7996 tomato plants. Ten plants were inoculated with each isolate and another ten with sterile 1X PBS (pH = 7.4) as a control. At 14 days post inoculation, both root and shoot tissues of treated and mock-treated plants were harvested. We calculated the plant growth response from the percent change in the weight of tissue of treated compared to mock-treated plants. Plant growth responses were determined by measuring fresh weight of both shoot and root. The growth response change of plants inoculated with the 21 selected isolates ranged from little change ($2.36 \pm 6.1\%$ change of fresh shoot weight) to vigorous ($38.96 \pm 4.97\%$ change of fresh shoot weight) stimulation of plant growth relative to the mock-treated plants (Figure 2.6, Appendix Figure A2.3). Overall, the growth promoting effects for a given isolate were similar between the root and shoot (Figure 2.6A and B).

Table 2.1. Isolates selected for the plant-binary interaction experiment. Three isolates were selected for every functional group. The PGPB *P. protegens* CHA0 of “Siderophore-Antagonism” group was used as a positive control for the experiment. Isolates were phylogenetically distinct from one another with the exception of two pairs of isolates, WV-94 with HA-28 and WV-44 with WV-162. Ten isolates were selected from each of the original hosts, H7996 and WV. Hf = High-frequency; WV ex = West Virginia exclusive; H996 ex = H7996 exclusive.

Functional Group	Isolate	OTU #	Genus	Family	Host	OTU class
Phosphorus	22	3	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	H7996	Hf
	104	28	<i>Bacillus</i>	<i>Bacillaceae</i>	WV	Hf
	121	40	<i>Microbacterium</i>	<i>Microbacteriaceae</i>	WV	WV ex
Auxin	44	11	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	WV	Hf
	94	5	<i>Enterobacter</i>	<i>Enterobacteriaceae</i>	WV	Hf
	195	54	<i>Xanthomonas</i>	<i>Xanthomonadaceae</i>	WV	WV ex
Siderophore	38	9	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	H7996	H7996 ex
	59	19	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	WV	WV ex
	109	37	<i>Sinorhizobium</i>	<i>Rhizobiaceae</i>	WV	WV ex
Antagonism	64	20	<i>Bacillus</i>	<i>Bacillaceae</i>	H7996	H7996 ex
	78	26	<i>Bacillus</i>	<i>Bacillaceae</i>	H7996	H7996 ex
	91	30	<i>Stenotrophomonas</i>	<i>Xanthomonadaceae</i>	H7996	H7996 ex
Auxin-Siderophore	28	5	<i>Enterobacter</i>	<i>Enterobacteriaceae</i>	H7996	Hf
	46	13	<i>Chryseobacterium</i>	<i>Flavobacteriaceae</i>	WV	WV ex
	162	11	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	WV	Hf
Auxin-Antagonism	89	29	<i>Rhizobium</i>	<i>Rhizobiaceae</i>	H7996	H7996 ex
	131	45	<i>Arthrobacter</i>	<i>Micrococcaceae</i>	H7996	H7996 ex
	182	52	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	WV	WV ex
Siderophore-Antagonism	129	44	<i>Bacillus</i>	<i>Bacillaceae</i>	H7996	H7996 ex
	141	48	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	H7996	Hf
	CHA0	+	<i>Pseudomonas</i>	<i>Pseudomonadaceae</i>	PGPB	PGPB

We first compared the impact of isolates within the same functional groups on fresh shoot weight using one-way ANOVA test followed by Tukey post-hoc test. As expected, the positive control *P. protegens* CHA0, which was categorized as being in the Siderophore-Antagonism group, significantly promoted shoot growth, with a $23.28 \pm 8.77\%$ response change of fresh shoot weight compared to mock-inoculated plants ($P < 0.05$). The three members of the group Siderophore-Antagonism, which are *P. protegens* CHA0, HA-129, and HA-141, stimulated plant growth in a similar rate to one another and did not significantly differ in their ability to promote plant growth ($P > 0.05$). This phenomenon could also be observed in isolates of the other six functional groups. For all groups, all three isolates categorized in the same *in vitro* functional group did not differ from one another in growth promotion ability as measured by root and shoot weight. Thus, although they were phylogenetically different from each other, members of each functional group similarly impacted plant growth.

Isolates in the three functional groups that promoted shoot growth, Auxin, Siderophore, and Antagonism, also significantly stimulated root growth ($P < 0.05$) (Figure 2.6, Appendix Table A2.3). Similar to the response rate of the shoot, the changes in fresh root weight of plants inoculated with isolates in two groups, Phosphorus and Auxin-Antagonism, were not significantly different compared to the mock-inoculated plants ($P > 0.05$). The isolates in the Auxin-Siderophore and Siderophore-Antagonism groups exhibited an intermediate plant phenotype in both shoot and root growth promotion. Together, these data show that isolates with similar *in vitro* functional traits have similar impacts on plant growth.

Isolates that were members of the Auxin or Antagonism group significantly enhanced both root and shoot weights ($P < 0.05$). However, the plant growth promotion effect was not observed in plants inoculated with isolates of the group with combined functions Auxin-Antagonism ($P > 0.05$) (Figure 2.6). A negative relationship between these two functional traits was also previously observed in the *in vitro* functional characterization experiment (Figure 2.4). We did not observe any differences between the presence of DAPG and HCN biosynthesis genes, and the response rate of inoculated plants (Appendix Figure A2.6). This may have been because these traits are relevant for plant growth promotion in the context of antibiosis against other microbes. Alternatively, the presence of the gene does not necessarily indicate production of an antimicrobial compound.

We next compared the effect of different functional groups on plant growth using Two-Way ANOVA test following by Tukey post-hoc test. Functional groups differentially impacted both shoot and root growth. For example, the phosphorus functional group had a significantly different impact on both root and shoot growth than the auxin and siderophore functional groups, but a similar impact on growth as the auxin-antagonism mixed functional group (Figure 2.6).

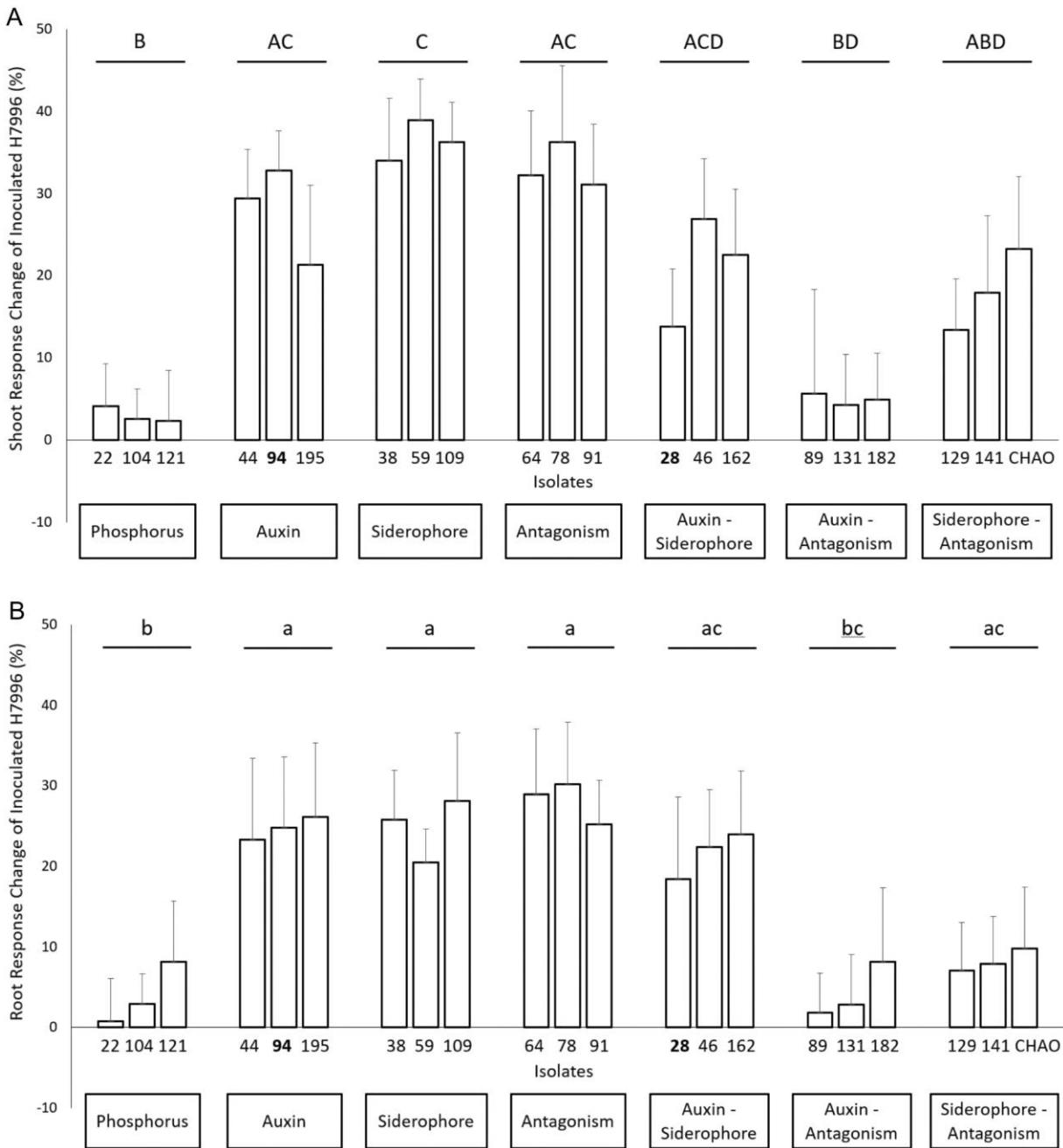


Figure 2.6. Isolates within the same functional group similarly impact the host phenotype, but functional groups differentially impact plants. Response rate was calculated for the change in (A) shoot or (B) root weight of inoculated H7996 compared with mock-treated plants of the same genotype. Bar plot showing the host response rate when inoculated with the selected isolates. Isolates with the same taxonomical classification, WV-94 of ‘auxin’ and HA-28 of ‘auxin–siderophore’ groups, are in bold. n=10 plants per isolate; different letters indicate significantly different at P<0.05 from Tukey’s post-hoc test. H7996, *S. lycopersicum* cv. Hawaii7996.

2.4.5 The effect of the isolates on plant growth is not explained by taxonomic classification or host origin

We next asked whether a specific bacterial family may effectively promote tomato growth. The 21 isolates selected for plant-binary interaction experiments included eight families: *Flavobacteriaceae*, *Bacillaceae*, *Microbacteriaceae*, *Micrococcaceae*, *Rhizobiaceae*, *Xanthomonadaceae*, *Pseudomonadaceae*, and *Enterobacteriaceae* (Table 2.1). Seven of the 21 selected isolates were members of the bacterial family *Pseudomonadaceae* and four of the 21 isolates belonged to the family *Bacillaceae*. We did not see differences in the plant growth promotion between the two families ($P > 0.05$) (Appendix Figure A2.7). This suggests that the taxonomic family of an isolate was not a factor in its effect on plant growth. We also investigated whether an isolate's host origin played a role in determining its plant growth promotion ability. We observed no significant difference between the plant growth promotion effect and an isolate's host origin ($P > 0.05$) (Appendix Figure A2.8). Further, we did not find any differences between high-frequency vs host-exclusive OTU and plant growth promotion effect ($P > 0.05$) (Appendix Figure A2.9). The impact of a bacterial isolate on plants in both shoot and root was not phylogenetically conserved (Blomberg's $K < 0.5$, Pagel's $\lambda < 1$, $P > 0.05$; Appendix Table A2.4, Appendix Figure A2.10). Together, these results suggested that for isolates within the same taxa, the impact on plant growth was better explained by their *in vitro* functional group classification than by their taxa. Consistent with this, two isolates with a shared OTU, WV-94 in the Auxin group and HA-28 in the Auxin-Siderophore group, were significantly different from one another in their impact on shoot weight ($P < 0.05$) (Figure 2.6 and Appendix Figure A2.11). Although both isolates promoted plant growth, the growth promoting effect of WV-94 was considerably stronger than HA-28 ($32.82 \pm 4.83\%$ change of fresh shoot weight vs. $13.82 \pm 7.06\%$ respectively).

Given that isolates within the same functional group impacted plant growth the same way, we next examined whether specific functional traits of an isolate were predictive of its effect on plant growth. Regression analysis showed that none of the individual functional traits correlated with an isolate's impact on plant growth (all $r < 0.25$, Appendix Figure A2.12 and Appendix Figure A2.13). Model selection based on the Akaike Information Criteria (AICc) indicated that no specific functional trait predicted an isolate's impact on plant phenotype (Appendix Table A2.5). Instead, models which contained the categorical predictor 'group' (describing an isolate's group classified from the *in vitro* functional characterization experiment) were more likely to explain plant

response (Appendix Table A2.5). These data suggest that multiple functional traits may be helpful in predicting the impact of bacteria on a host plant.

2.5 Discussion

2.5.1 *In vitro* functional traits of root endophytes are informative for isolates' in planta impact

We demonstrate that multiple functional traits are informative for understanding the impact of bacterial root endophytes on plant growth. Such traits were more relevant for plant growth than OTU classification, or tomato host. We note that the Z-score used for our functional groups indicates which functions are relatively stronger than others within a given isolate. However, other functions are also present, and may also contribute to plant growth promotion. Root endophytes that produced high levels of IAA, siderophores, or were highly antagonistic to *R. solanacearum* all significantly promoted plant growth. Auxin and siderophore production are both well described features of plant growth promoting bacteria (Sayyed and Patel, 2011). It is less clear why bacteria in the high antagonism group promoted plant growth. These microbes may produce another plant growth promoting compound that we did not assay or were able to outcompete other root-associated microbes.

Root endophytes that were able to solubilize inorganic phosphate very well had little impact on growth. Although phosphate is a crucial macronutrient important for normal plant physiological processes, excessive application of inorganic phosphate fertilizer has been observed to irreversibly alter the soil microbiome and adversely impact plant growth (Kaminsky et al. 2018). Thus, we may not expect bacteria with strong phosphate metabolizing function to promote plant growth in our experiments.

We were intrigued by the observation that microbes in the “Auxin-Antagonism” group did not promote plant growth, because isolates in each of the one-function groups “Auxin” and “Antagonism” significantly promoted plant growth. “Auxin” and “Antagonism” were negatively correlated with one another in the *in vitro* experiment – in general, isolates that produced high levels of auxin were not antagonistic to *R. solanacearum*, and vice versa. A small number of microbes both produced auxin and were moderately antagonistic to *R. solanacearum*. Although the effect of bacterial IAA on plant-microbe interactions has been extensively studied, its influence on bacteria-bacteria interactions remains largely unexplored (Spaepen, Vanderleyden, and Remans

2007). Microbial-produced IAA from other plant-associated bacteria inhibits the synthesis of the antibiotic abdrimid of the rhizobacterium *Serratia plymuthica* A153 (Matilla et al. 2018). *Variovorax* strains degrade auxin in the rhizosphere, which attenuates the root growth inhibition caused by some rhizobacteria (Finkel et al. 2020). It is possible there is a link between the ability of the endophytes to produce IAA and their antagonistic activity against *R. solanacearum*. We speculate that the microbial genomes which contain the genes to produce IAA may not contain those needed to produce the inhibitory compounds. Alternatively, there may be competition for the same metabolites used in the synthesis of both IAA and the inhibitory compound, or expression of the genes required for both outputs may not be possible, making it challenging for microbes to produce both at the same time. It is also possible that our auxin screening assay is not sufficiently sensitive to capture lower levels of auxin production, and the correlation between IAA production and antagonistic traits may change when examining lower levels of IAA. Future work is needed to understand the relationship between these two functions, the nature of the inhibitory compound(s), whether their antagonistic activity is effective against other microbes and whether it is maintained during root colonization.

We observed a positive correlation between isolates with genomes that contain the *phlD* gene, which encodes the antimicrobial metabolite DAPG, and the ability to produce siderophores. The antagonistic activity of pseudomonads against pathogens may be the result of synergistic actions of both DAPG and siderophores (Cronin et al. 1997). HPLC analysis suggested that the antibiosis of two strains of *Pseudomonas* spp RS-9 and J12 against *R. solanacearum* is partially due to the activity of DAPG (Rai, 2017; Zhou et al., 2012). We did not find a correlation between antagonism against *R. solanacearum* and the production of DAPG. This could be due to differences in the strain of *R. solanacearum* used in our work and those of (Zhou et al., 2012) and (Rai 2017).

We choose the traits here because each was known to be a feature of plant growth promoting rhizobacteria (Hayat et al. 2010b; Souza et al. 2015), and each trait could be assayed with a low-cost, relatively fast and straightforward experiment with established protocols. Testing additional traits of rhizobacteria, such as cytokinin or ethylene production, or using assays with increased sensitivity, may improve our ability to predict which plant growth promoting bacteria should be investigated further for *in planta* experiments.

2.5.2 Bacterial phenotypes are independent of OTU classification

Understanding the relationship between microbial function and taxonomic classification is helpful for the practical application of microbial inoculants, including their use in crop production. While in general there tends to be conservation between phylogeny and trait (Amend et al. 2016; Isobe et al. 2020; Morrissey et al. 2016), processes like convergent evolution, gene loss, and horizontal gene transfer can tangle the relationship between phylogeny and trait (Doolittle 1999; Snel, Bork, and Huynen 2002). In addition, the relationship between phylogeny and function varies depending on trait, taxonomic rank, and habitat (Goberna and Verdú 2016; Martiny et al. 2015; Martiny, Treseder, and Pusch 2013; Morrissey et al. 2019; Philippot et al. 2010). High complexity traits – which are controlled by multiple genes – have higher trait depth and tend to be conserved at deeper taxonomic ranks (Martiny et al. 2015, 2013). In contrast, traits of lower complexity (i.e. controlled by one gene), have lower trait depth and are often present in multiple, shallow clades. At high taxonomic resolution (species and genera), like in our study, there is generally less correspondence between phylogeny and function (Jaspers and Overmann 2004). Habitat can also impact the relationship between phylogeny and function, since distinct environments may require specific microbial functional profiles, but vary in taxonomic composition (Cheaib et al. 2018; Isobe et al. 2019, 2020; Louca, Parfrey, and Doebeli 2016).

Consistent with previous studies showing that closely related taxa have different growth rates and functional traits (Hahn et al. 2016, 2021; Jaspers and Overmann 2004; Lladó Fernández, Větrovský, and Baldrian 2019), we found that isolates within the same OTU had different *in vitro* functions and effects on plant growth production, and isolates in different OTUs had similar functions. Thus, our results suggest that the effect of an isolate on plant growth is independent of phylogeny. However, had we used a different set of traits, focused on another taxonomic rank, or included a wider phylogenetic range of bacteria, we may have found different results. Additionally, the expression of many microbial traits is dependent on microbial population size (Escalas et al. 2019), and it is not clear whether the *in vitro* phenotypes we measured are also apparent during root colonization.

We also found that host origin was not predictive of an isolate's phenotype. This could be due to the specialized habitat from which our endophytes were derived, or because we used two plant hosts in the same genus. Bacteria in our study were derived from the root endosphere, an environment which exerts unique pressures on microbes (Bulgarelli et al. 2012). Successful

colonization of the root endosphere may require the expression of specific traits, regardless of taxonomic classification.

Our research demonstrates that more work is needed to understand the relationship between microbial taxa and function. Production of DAPG is well-studied in the pseudomonads for suppression of root pathogens (Keel 1992; Meyer et al. 2016) and is thought to be an ancestral trait of pseudomonads (Moynihan et al. 2009). However, our results indicate that the presence of *phlD* gene is not exclusive to pseudomonads and support a recent study that identified the *phl+* cluster outside of the *Pseudomonas* genus (Almario et al. 2017). Further studies investigating the phylogenetic depth of DAPG biosynthesis pathway in bacteria are necessary to gain better understandings of phylogenetic conservation of this trait.

2.5.3 The potential for developing economic and effective strategies to promote crop performance in agriculture with microbial inoculants

Using microbial inoculants, or biofertilizers, has been a strategy to boost crop performance for over 100 years (Jack et al. 2021; Kaminsky et al. 2019; Santos, Nogueira, and Hungria 2019), but has not proven as promising in the field as in the lab or greenhouse (Hawkes and Connor 2017; Sessitsch, Pfaffenbichler, and Mitter 2019). Our study suggests that developing inoculants based on functional traits may help promote success in the field. However, our study has several limitations. We conducted our plant-binary interaction experiments in the greenhouse, and we limited the host-microbe interaction factor to a single isolate and one tomato genotype per sample. Bacteria traits can depend on the surrounding environment, and it is not clear whether the functions we quantified are also present *in planta*, nor is it clear whether they would contribute to plant growth promotion in a microbial community. We choose functions that are known to promote plant growth, but assaying additional functions may be more informative.

Together our results demonstrate that easily screened *in vitro* functional traits are informative for plant growth in greenhouse-based assays. Although more work is needed to determine whether these traits are also predictive of plant performance in more complex communities and environments, this provides an initial framework to test additional microbes and develop synthetic communities for improved crop production.

2.6 References

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CHAPTER 3. TOMATO GENOTYPE MODULATES SELECTION AND RESPONSES TO ROOT MICROBIOTA

3.1 Abstract

Using microbial inoculants to enhance plant health is promising for crop improvement. However, for success, knowledge of how different cultivars within a crop species select and respond to the root microbiome is critical. The aims of this study were to 1) determine the contribution of tomato genotype to the tomato root bacterial microbiome, and 2) investigate whether closely related tomato genotypes differ in their selection of, and response to, root endophytes. We used 16S rRNA amplicon sequencing to examine the root bacterial communities of six *Solanum lycopersicum* (domesticated tomato) and two *Solanum pimpinellifolium* (wild tomato) accessions. We found that across tomatoes, both the root endosphere and rhizosphere were impacted by genotype. Genotype accounted for 10% of the variation in root microbiota. Two bacterial families, *Bacillaceae* and *Rhizobiaceae*, were significantly enriched in the root endosphere in at least six of the eight tomato genotypes. To investigate whether closely related tomato genotypes differed in selection of these endosphere-enriched taxa, we profiled the root endosphere of 20 recombinant inbred lines (RILs) derived from two of the genotypes. The abundance of *Bacillaceae* and *Rhizobiaceae* varied quantitatively in the root endosphere of the RILs. Inoculation of 16 RILs with a *Bacillaceae* isolate identified from the root endosphere of field grown tomatoes showed that RIL responses, in terms of shoot and root growth, varied from less than 5% growth enhancement to over 40%. Our data show that tomato genotypes have distinct but overlapping root bacterial microbiomes and respond differently to specific bacterial endophytes.

3.2 Introduction

Plant roots are intimately connected to a diversity of microbes in the soil. Some soil microbes are root pathogens and cause destructive diseases in their hosts. Others are beneficial root colonizers and contribute to plant health and function through enhancing root growth and nutrient acquisition, promoting defense responses, or augmenting abiotic stress tolerance (reviewed in Berendsen et al. 2012; Bulgarelli et al. 2013; Pieterse et al. 2014). Optimizing host

selection of root-associated beneficial microbial communities holds promise for sustainably increasing crop production. However, to fulfill this promise, understanding how crop genetic variation shapes root microbial communities is needed. Here we asked how genetic variation among cultivated and wild tomatoes impacts selection of the root bacterial microbiome. We hypothesized that closely related tomato genotypes would differ in their selection of, and responses to, soil microbiota.

Root microbiome structure is determined by soil characteristics, root compartment, and plant genotype (Berendsen et al. 2012; Bulgarelli et al. 2013; Pieterse et al. 2014). Root compartments - the rhizosphere, rhizoplane (root surface) and root endosphere (inside the root) each act as microhabitats and have a distinct assemblage of microbes (Edwards et al. 2015; Fitzpatrick et al. 2018; Lebeis et al. 2012; Lundberg et al. 2012; Schlaeppi et al. 2014; Lee et al. 2019; Poudel et al. 2019). Multiple studies have demonstrated higher levels of bacterial diversity within the rhizosphere compared to the endosphere (Lundberg et al. 2012; Lebeis et al. 2012; Edwards et al. 2015; Fitzpatrick et al. 2018; Lee et al. 2019; Poudel et al. 2019). Current models of root microbiome assembly suggest a multi-step model in which each root compartment selects a subset of microbes from the surrounding compartment, resulting in lower diversity in the root endosphere compared to rhizosphere (Edwards et al. 2015; van der Heijden and Schlaeppi 2015; Reinhold-Hurek et al. 2015).

Although the effect of soil type on root microbiome structure is greater than that of root compartment and plant genotype (Lundberg et al. 2012; Schlaeppi et al. 2014, Lebeis et al. 2015; Yeoh et al. 2017; Chen et al. 2018; Veach et al. 2019) elucidating the role of plant genotype is important due to the potential to select for plants which recruit specific microbial taxa or functions. Evidence from *Arabidopsis* and maize supports the possibility of genetic control of host microbiome selection. Quantitative trait loci (QTL) for selection of phyllosphere microbiome taxa have been identified in *Arabidopsis* (Horton et al. 2015) and maize (Balint-Kurti et al 2010). Maize GWAS analysis suggests that host genetics impact leaf microbiome metabolic functions (Wallace et al. 2018). Plant selection for root microbial taxa may be due to traits that influence the root microbiome including root exudates (Carvalhais et al. 2015; Stringlis et al. 2018, Hu et al. 2018; Zhelnina et al. 2018), and plant hormones (Long et al. 2010; Doornbus et al. 2011; Carvalhais et al. 2013; 2015; Lebeis et al. 2015; Liu et al. 2017; Veach et al. 2019).

The effect of host plant genotype on the root microbiome is evident both between (Fitzpatrick et al. 2018; Schlaeppi et al. 2014; Bulgarelli et al. 2015; Zachow et al. 2014) and within plant species (Lundberg et al. 2012; Peiffer et al. 2013; Cardinale et al. 2015; Poudel et al. 2019; Sharaf et al. 2019), and within root compartments (Lundberg et al. 2012; Lebeis et al. 2015; Edwards et al. 2015), although is not universal (Wagner et al. 2016).

Consistent with the idea that plant genotype alters root microbiome selection, different plant genotypes may have distinct responses to the same microbe. Understanding how different genotypes respond to the same root endophyte is important for effectively using bacterial taxa to promote crop production. For example, tomato recombinant inbred lines (RILs) differed in their response to the biocontrol strain *Bacillus cereus* UW85 (Smith et al. 1999). Arabidopsis accessions differed in fresh weight and root architecture after treatment with *P. simiae* WCS417r (Haney et al. 2015) and GWAS led to the identification of several candidate genes for response to WCS417r (Wintermans et al. 2016). In Arabidopsis, GWAS identified a cytochrome P450 gene, *CYP71A*, that regulates responses to *Pseudomonas* sp. CH267. The two alleles of *CYP71A* resulted in differences in the ability of plants to gain fresh weight in response to CH267 (Koprivova et al. 2019). Plant responses to root microbiota thus appear to be genetically encoded.

Here we use eight tomato genotypes, including six genotypes of cultivated tomato *S. lycopersicum*, and two accessions of wild tomato *S. pimpinellifolium*. We also include 20 RILs derived from one of the cultivated and one wild type tomato. We ask how selection of root bacterial communities varies across tomato genotypes and how different tomato genotypes respond to inoculation of an isolate identified from the root endosphere of one cultivar. Tomato is the second most important vegetable crop globally (in terms of tons produced) (FAO, 2018). Questions of microbiome selection and responses are important for developing microbiome management strategies, particularly in tomato, in which a multitude of different cultivars are used by commercial growers, the seed industry, small scale farmers and home gardeners. Our results suggest that even closely related tomato genotypes select for different, but overlapping, microbial assemblages and have distinct responses to a microbial inoculant.

3.3 Materials and Methods

3.3.1 Soil mix

Soil mix was prepared by hand-mixing autoclave-sterile potting mix and field soil in a 2:1 ratio by volume. The field soil was a sandy loam collected from the top 10 cm of a conventional agricultural field at Throckmorton Purdue Agricultural Center (40.2° N, 86.9° W) in three batches from April – June 2017, ground, sieved to 4 mm, air dried at 27 °C to a constant weight, and mixed to homogenize the three batches. Potting mix was Fafard germination mix, custom blend with 56.69% sphagnum peat moss, composted bark, perlite, vermiculite, dolomite lime, wetting agent and 0.001% silicon dioxide (SKU code 8269028, lot Q17.05). The potting mix was autoclaved for 30 min at 122.8 °C. Samples of field soil and potting mix/field soil mixture were sent to A&L Great Lakes Laboratories for nutrient characterization (Appendix Table B3.1). Three technical replicates were performed for each soil sample.

3.3.2 Plant genotypes

To investigate the effect of tomato genotype on the root microbiome, the following genotypes were used: *S. lycopersicum* cvs. Moneymaker (MM), Bonny Best (BB), Pearson, Castlemart II (CMII), UC82B, and Hawaii7996 (H7996), *S. pimpinellifolium* accessions LA2093 and West Virginia700 (WV), and 20 recombinant inbred lines (RILs) derived from H7996 and WV. MM, BB and Pearson are heirloom fresh market tomato varieties. CMII and UC82B are processing tomatoes. H7996 and WV are the parents of a well-described RIL population that has been used to identify QTL for resistance to the soilborne pathogenic bacterium *Ralstonia solanacearum* (Wang et al. 2000; Carmeille et al. 2006). LA2093 has been described for its resistance to plant pathogens (Caldwell and Iyer-Pascuzzi 2019; Ashrafi et al. 2009). RILs were in the F9 generation. Microbiome analyses of all genotypes was performed together as described below.

3.3.3 Plant growth and harvest for microbiome analyses

Seeds were surface sterilized by incubating with gentle rocking in 50% bleach for ten minutes and then rinsing 5-6 times in sterile ddH₂O. Seeds were then stored at 4 °C overnight. Surface-sterile seeds were planted in randomized complete blocks in 36-pot flats with 1 unplanted

cell per block to represent the bulk soil control. Three seeds were planted to each pot and thinned to one plant after germination. Eight full biological replicate blocks were planted to account for any issues with germination. Flats were fertilized once per week with 500 mL of 150 ppm Nitrogen standard MiracleGro fertilizer. Plants were grown in a light and temperature controlled greenhouse (temperature setting 75-84°F). Lights operated on a 16:8 hour day cycle.

Four blocks were harvested for sequencing after seedlings reached 4-leaf stage (approximately 2.5 weeks). Due to filtering for low quality sequencing results (<2000 reads/sample; see results), all genotypes have either three or four replicates. Rhizosphere and endosphere samples were collected from each genotype except RILs, for which only endosphere samples were collected (see Appendix Table B3.2 for all genotypes). For rhizosphere sampling, roots were removed from the pot and excess soil was removed gently under aseptic conditions until only soil within 1 mm from the root surface remained (Lundberg et al. 2012). Roots were then placed into 15 mL conical tubes containing sterile 1X PBS, shaken manually, and then placed in a new 15 mL conical tube for surface sterilization. Conical tubes containing the rhizosphere soil were spun at 5000 rpm for 5 min. Excess liquid was decanted, and soil pellets were resuspended and transferred to a 1.5 mL sterile Eppendorf tube. The Eppendorf tube was spun at max speed for 10 min, supernatant decanted, rhizosphere soil frozen in liquid nitrogen (LN₂) and stored at -80 °C until DNA extraction. These samples were designated the rhizosphere compartment samples. Roots were cleaned by performing an additional rinse to remove any remaining soil. Subsequently, roots were surface sterilized by incubating in 5% bleach with gentle shaking for 2 min, then rinsed 3 times in sterile ddH₂O before freezing in LN₂ and storing at -80 °C until DNA extraction. These samples were designated the endosphere compartment samples.

3.3.4 Root architecture and disease resistance phenotyping in RIL population

The roots of remaining RIL plants that were not harvested for 16S rRNA sequencing were harvested by gently removing excess soil first by hand, and then remaining soil was removed by gently rinsing in a water-filled tub and gently brushing with a paintbrush to prevent root breakage. Roots were then imaged on a flatbed scanner and root architecture traits were analyzed with WinRhizo (regular, v.2016). Phenotypic data on resistance of the 20 RIL lines to *Ralstonia solanacearum* were collected in a separate experiment (Appendix Table B3.2). For this, *R. solanacearum* was soil soak inoculated at 10⁸ CFU/g soil as in French et al. 2018. Wilting was

assessed as the percentage of leaves wilting at 8 days post inoculation. The average wilting and root architecture values from 4 – 5 individuals of each line were then compared to average values of bacterial community composition traits for each RIL via simple regression.

3.3.5 DNA extraction and library preparation

Frozen roots were ground under liquid nitrogen (LN₂) in sterile mortars and pestles before DNA extraction. Each root sample weighed approximately 0.22 ± 0.03 g. DNA was extracted from all samples using Norgen Soil DNA (Norgen Biotek Corp, Canada) extraction kits. DNA concentration and purity were measured by Nanodrop3000. Library preparation was performed using the Illumina 16S Metagenomics Sequencing Library Preparation protocol according to the manufacturer's instructions with slight modifications. Two step PCR was performed to amplify the V5 through V7 region of the 16S rRNA gene with the chloroplast excluding primer pair 799F-1193R (Chelius and Triplett 2001; Beckers et al. 2016) and to add Illumina Nextera XT indices.

First, all DNA samples were standardized to 5 ng/μL. One negative water control and one mock community DNA control (ZymoBIOMICS Microbial Community Standard, Zymo Research, Irvine, CA, USA) were included on each plate. 25 μL PCR reactions were performed with 2.5 μL genomic DNA, 12.5 μL 2X KAPA HiFi HotStart ReadyMix and 5 μL each of the forward and reverse primers (1 μM) in two 96 well plates. Primer sequences with adapters were as follows: 799F + Nextera adapter:

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACMGGATTAGATACC-
CKG-3' and 1193R + Nextera adapter: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-
CAGACGTCATCCCCACCTTCC-3', which produces a ~480 bp product. Underlined portions indicate the 16S primer portion. PCR cycle performed as follows: 95 °C for 3 min, [95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec] x 27 cycles, 72 °C for 5 min.

After the initial PCR step, PCR products were run on 1.5% agarose gels. The ~480 bp band was excised and gel extracted with the Invitrogen PureLink gel purification kit. This step was performed to exclude the larger mitochondrial band amplified by the 16S primers. Gel purified PCR products were used for the second PCR step to attach dual indices and sequencing adapters. For this step, 50 μl PCR reactions were performed with 5 μL purified Step 1 PCR product, 25 μL 2X KAPA HiFi HotStart ReadyMix, 10 μL of sterile ddH₂O and 5 μL each of the Index 1 and Index 2 Nextera XT Primers (set A and B) in two 96 well plates. PCR cycle was as follows: 95 °C

for 3 min, [95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec] x 8 cycles, 72 °C for 5 min. Standard AmpureXP bead purification was performed on the Step 2 PCR products.

Step 2 PCR products were quantified at the Purdue Genomics Core by mixing 1 µL of each library into a pool and sequencing as 10% of a MiSeq paired end 250 bp run. The library sizes were estimated from the number of reads obtained from each library and used to calibrate library concentrations for the final pool. All 188 samples were multiplexed into a single pool in equivalent concentrations. The pool was run on an Agilent bioanalyzer chip to confirm library size and purity. The pool was sequenced at the Purdue Genomics Core Facility using Illumina MiSeq V2 chemistry with paired end 250 bp sequencing.

3.3.6 Sequence processing

Demultiplexing was performed by the Purdue Genomics Core with Illumina software; adapter removal and primer clipping was performed with Trimmomatic (v 0.36) (Bolger et al. 2014) and Cutadapt (v 1.13) (Martin 2011). All subsequent processing was performed using packages in R (v 3.5.0) and Bioconductor (v 3.7). Reads were processed through the DADA2 (v 1.8.0) pipeline by filtering and trimming based on read quality, inferring error rates, merging paired end reads, removing chimeras, and assigning taxonomy with the Silva reference database v. 132 (Callahan et al. 2016). Likely contaminant sequences were removed with the decontam package using negative controls to infer likely contaminants (Davis et al. 2018). Very low abundance sequences (fewer than 2 reads in 10% of the samples) were removed. The average number of reads per sample was 10,612 reads. The median number of reads was 9,181. Archaea represented a very low proportion of reads (approximately 350 reads) and we would not have been able to draw any meaningful conclusions by including them. Thus, they were filtered out along with chloroplast and mitochondrial contaminant reads. Samples with α -diversity measurements more than 1.5X outside the interquartile range were considered outliers and removed. α -diversity measurements performed with the Phyloseq (v 1.24.0) package after subsampling to the smallest library size (2,569 reads) 100 times and averaging the results (McMurdie and Holmes 2013). β -diversity measurements and constrained analysis of principle coordinates (CAP) were performed with Phyloseq and vegan (v 2.5-2) packages with reads proportionally scaled to the smallest library size (code courtesy of Deneff lab tutorial - <http://deneflab.github.io/MicrobeMiseq/>). Normalization and differential abundance analysis were performed with DESeq2 (v 1.20.0) (Love et al. 2014). Linear

regression performed with `lm()` function in R. All plots were made with the `ggplot2` (Wickham 2009) package and arranged in Inkscape (v 0.92.3). All code for analysis and figure generation can be found in the Purdue University Github to <https://github.rcac.purdue.edu/AnjaliIyerpascuzziGroup/Tomato-Root-16S-Sequencing> as ‘Tomato-Root-16S-Sequencing’. Sequencing summary is listed in Appendix Table B3.3.

3.3.7 Identification of culturable root endosphere microbes

H7996 and WV were planted in May 2017 at Throckmorton Purdue Agriculture Center (TPAC), located (40.2° N, 86.9° W). In August, roots were harvested, washed and sterilized as above. Root tissue was then homogenized in a sterile mortar and pestle with sterile 1X PBS. 100mL of homogenized liquid was dilution plated on to three different types of media: Luria-Bertani (LB) for isolation of fast-growing copiotrophic bacteria, R2A for isolation of slow-growing oligotrophs, and King’s B Medium for Pseudomonads. 183 isolates were collected and identified by Sanger sequencing of the 16S rRNA subunit. The bacterial colony was gently picked and diluted in 100μL ddH₂O. 20μL colony PCR reaction was performed with 10μL Apex Taq RED Master Mix 2.0X 1.5mM MgCl₂ (Genesee Scientific, San Diego, California, USA), 6μL sterile ddH₂O, 2μL bacterial cell solution, and 1μL each of the forward and reverse primers (1μM). Two 16S rRNA universal primers 27F and 1392R were used. Primer sequences were as follows:

27F: 5’-AGA GTT TGA TCM TGG CTC AG-3’

1392R: 5’-ACG GGC GGT GTG TAC A-3’

The PCR cycle for amplification was performed as followed: 94 °C for 5 min, [94 °C for 30 sec, 51 °C for 30 sec, 72 °C for 1.5 min] x 35 cycles, and 72 °C for 10 min. After the amplification step, PCR products were enzymatically purified with ExoSAP-IT™ PCR Product Cleanup Reagent using standard cleanup protocol from the manufacturer (Thermo Fisher Scientific). Purified PCR product was sent for Sanger sequencing and aligned to the NCBI database.

3.3.8 Plant growth promotion assays

Eighteen plants of each of 16 RILs derived from H7996 and WV, as well as 18 individuals of H7996 and WV were sown in autoclaved Metro Mix propagation mix and grown in the same

greenhouse under similar water and temperature conditions as the plants used for root microbiome sequencing. Seeds were surface sterilized one day before sowing in sterile soil. Plants were organized in a random complete block design with nine blocks; both treated and mock-treated plants of all genotypes were included in each block. At 10 days old, nine seedlings of each genotype were inoculated with a *Bacillus* isolate by pouring the bacteria over the soil to achieve 10^6 CFU/g soil. The isolate was originally identified from the root endosphere of H7996 tomato roots grown in field soil. This isolate was 97.1% identical to the high-frequency colonizing ASV 397. Another nine seedlings of each RIL and the parents were inoculated with the buffer 1X PBS (pH = 7.4) as controls. Plants were fertilized with Peter's Excel 15-5-15 Cal Mag at 1.6g/plant two days after inoculation. All nine blocks were harvested at 24 days old. For each genotype, plant growth promotion was determined by the effect of the isolate on fresh root and shoot weight compared to mock-inoculated control.

3.3.9 Data analysis for plant growth promotion assay

Fresh shoot and root tissues were weighed upon harvesting. The plant growth promotion effect was measured by response rate, which was calculated by the change in weight of the inoculated plant compared to the mock-treated plant of the same genotype within the same block. In total, nine response rates per genotype were obtained from nine blocks. Data normalization and analysis was performed in R (v 3.5.0). Response rate data was normalized using the formula $\ln(\text{response rate} + 2)$. One-way ANOVA and Tukey's test were performed on normalized response rate using the `aov()` and `TukeyHSD()` functions. Bar graphs were made with `ggplot2` package.

3.4 Results

3.4.1 The structure of the tomato root microbiota

To investigate the structure of the tomato root bacterial microbiome, we sequenced the V5-V7 region of the 16S rRNA from our samples by paired-end 250 bp MiSeq Illumina sequencing, resulting in 2.9 million high quality sequences after quality filtering and removal of chimera, non-target (mitochondria, chloroplast, archaea), and likely contaminant sequences (see Materials and Methods and Appendix Table B3.3). These sequences corresponded to 22,078 amplicon sequence variants (ASVs). We filtered for low abundance ASVs (fewer than 2 reads in less than 10% of the

samples), samples with fewer than 2000 total reads, and outliers. Our final data set for analysis consisted of approximately 1.4 million reads and 901 ASVs with an average of 10,612 reads per sample (see Materials and Methods for description of sequence processing; Appendix Tables B3.4-6 for raw ASV counts in each sample, sample data, and taxonomy).

We first examined the taxonomic structure of root bacterial communities in all tomato genotypes combined. Combining all genotypes, taxonomic classification of all 901 ASVs revealed 16 phyla with 7 phyla contributing to the majority of the bacterial microbiome (Figure 3.1; specific taxa are listed in Appendix Table B3.6). Proteobacteria are split into classes because they made up the majority of sequences across the dataset (>70%). Taxonomic classification of the ASVs at phylum level revealed that in the root rhizosphere, Gamma-proteobacteria (44.2%), Alpha-proteobacteria (22.7%), Actinobacteria (15.6%) and Delta-proteobacteria (4.1%) are most abundant, regardless of genotype. In the root endosphere, Proteobacteria are also common, and comprise about 70% of the abundance of most genotypic communities (Gamma- 46.6%, Alpha- 22.5%, and Delta- 3.4%). However, in contrast with the rhizosphere, Firmicutes (6.8%), Actinobacteria (15.0%) and Bacteroidetes (2.6%) compose the rest of the community.

The 16 phyla were comprised of 122 families (Appendix Table B3.6). Independent of tomato genotype, 39 families were differentially abundant between the rhizosphere and endosphere, including *Bacillaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, and *Rhizobiaceae* (Figure 3.2). Differential abundance results, independent of genotype, for phylum and family levels are shown in Appendix Tables B3.7-8 (differential abundance results by genotype are in Appendix Tables B3.9-16). Families that were abundant across all compartments included *Burkholderiaceae* (31.2%), *Sphingomonadaceae* (6.0%), *Caulobacteraceae* (4.0%), *Xanthobacteraceae* (3.8%), and *Pseudomonadaceae* (3.3%).

3.4.2 Impact of tomato genotype on the tomato root bacterial microbiome

Measurements of alpha (α)-diversity (richness and Shannon Index) revealed lower levels of alpha diversity in the root endosphere compared to the rhizosphere regardless of genotype (ANOVA, $F_{1,37} = 79.502$, $P < 0.001$) (Figure 3.3A, Appendix Figure B3.1, Appendix Table 3.17). This is consistent with other studies demonstrating lower diversity, or a ‘gating’ effect, in the root endosphere (Bulgarelli et al 2015; Schelappi et al; 2014; Lebeis et al 2015; Lundberg et al 2012; Edwards et al. 2015; Fitzpatrick et al. 2018; Poudel et al. 2019). There were no significant

differences in either richness nor Shannon Diversity among the tomato genotypes (ANOVA, $F_{7,37} = 1.592$, $p = 0.169$; average α -diversity in Appendix Table B3.17).

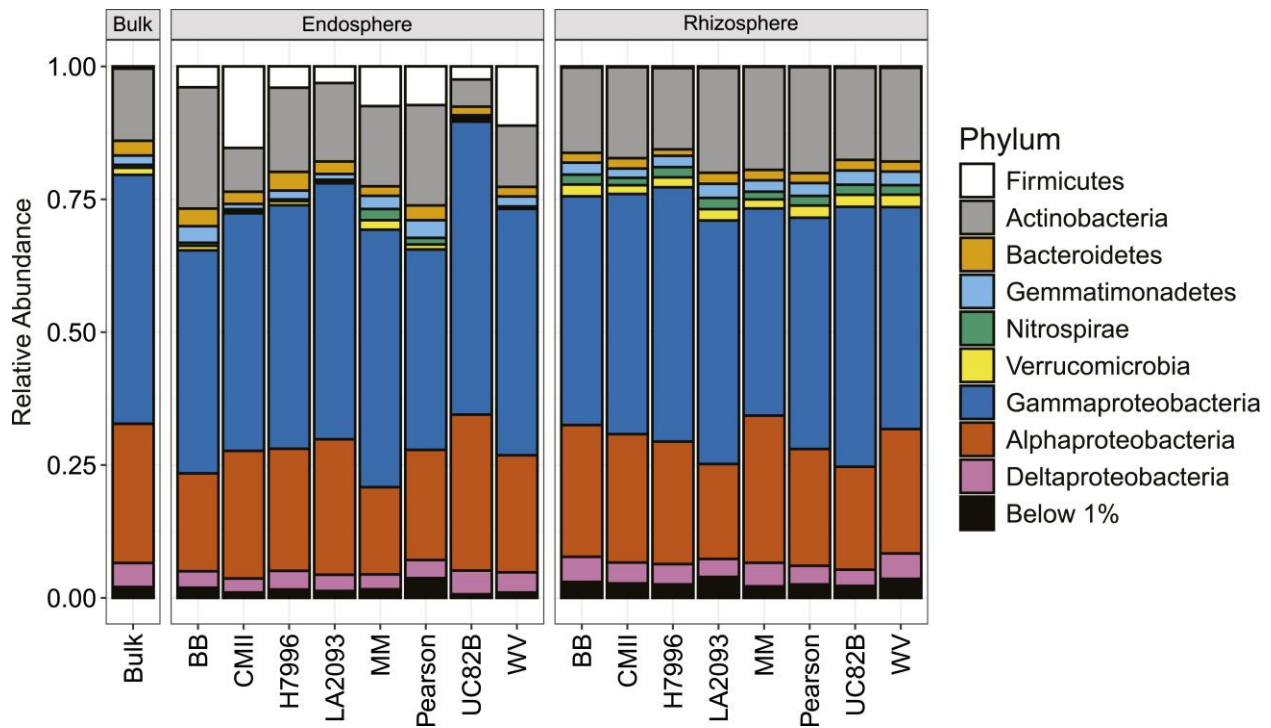


Figure 3.1. Tomato root microbiome bacterial community composition across compartments at the phylum level. Stacked bar plot of relative abundance of bacterial taxa at phylum level for each genotype. Proteobacteria comprised greater than 70% of the overall dataset and so were split into classes. Phyla with less than 1% average abundance across genotypes were grouped together and represented as “Below 1%”. Abbreviations: CMII = *Solanum lycopersicum* ‘Castlemart II’, WV = *S. pimpinellifolium* accession WestVirginia700, UC82B = *S. lycopersicum* ‘UC82B’, MM = *S. lycopersicum* ‘Money Maker’, BB = *S. lycopersicum* ‘Bonnie Best’, Pearson = *S. lycopersicum* ‘Pearson’, LA2093 = *S. pimpinellifolium* accession LA2093, and H7996 = *S. lycopersicum* ‘Hawaii7996’.

Bray-Curtis beta (β -) diversity patterns revealed a significant contribution of both microhabitat and genotype. We observed separation between the endosphere samples and the rhizosphere/bulk samples along the first axis (27%) and separation between the rhizosphere and bulk soil samples along the second axis (8.8%) (Figure 3.3B). Compartment accounted for 30% of overall variation (PERMANOVA, compartment: $F(2, 59) = 13.18$, $p < 0.001$). This compartmental specialization is consistent with other root microbiome studies (Bulgarelli et al. 2012; Lundberg et al. 2012; Lebeis et al. 2015; Edwards et al. 2015). Tomato genotype was responsible for approximately 10% of the variation (PERMANOVA, genotype, $F(7, 59) = 1.31$, $P = 0.048$), and there was a significant interaction between compartment and genotype (PERMANOVA, compartment:genotype, $F(7, 59) = 1.32$, $P = 0.041$). Distance among samples was higher within

the endosphere than the rhizosphere (Figure 3.3B). Dispersion analysis revealed significant differences among compartments ($F(2, 59) = 99.57, P < 0.001$), with all three compartments having significantly different dispersions from one another by Tukey's honest significant differences ($p < 0.05$). Canonical Analysis of Principle Coordinates (CAP) to examine the role of compartment and genotype on bacterial community diversity revealed similar results (Appendix Figure B3.2).

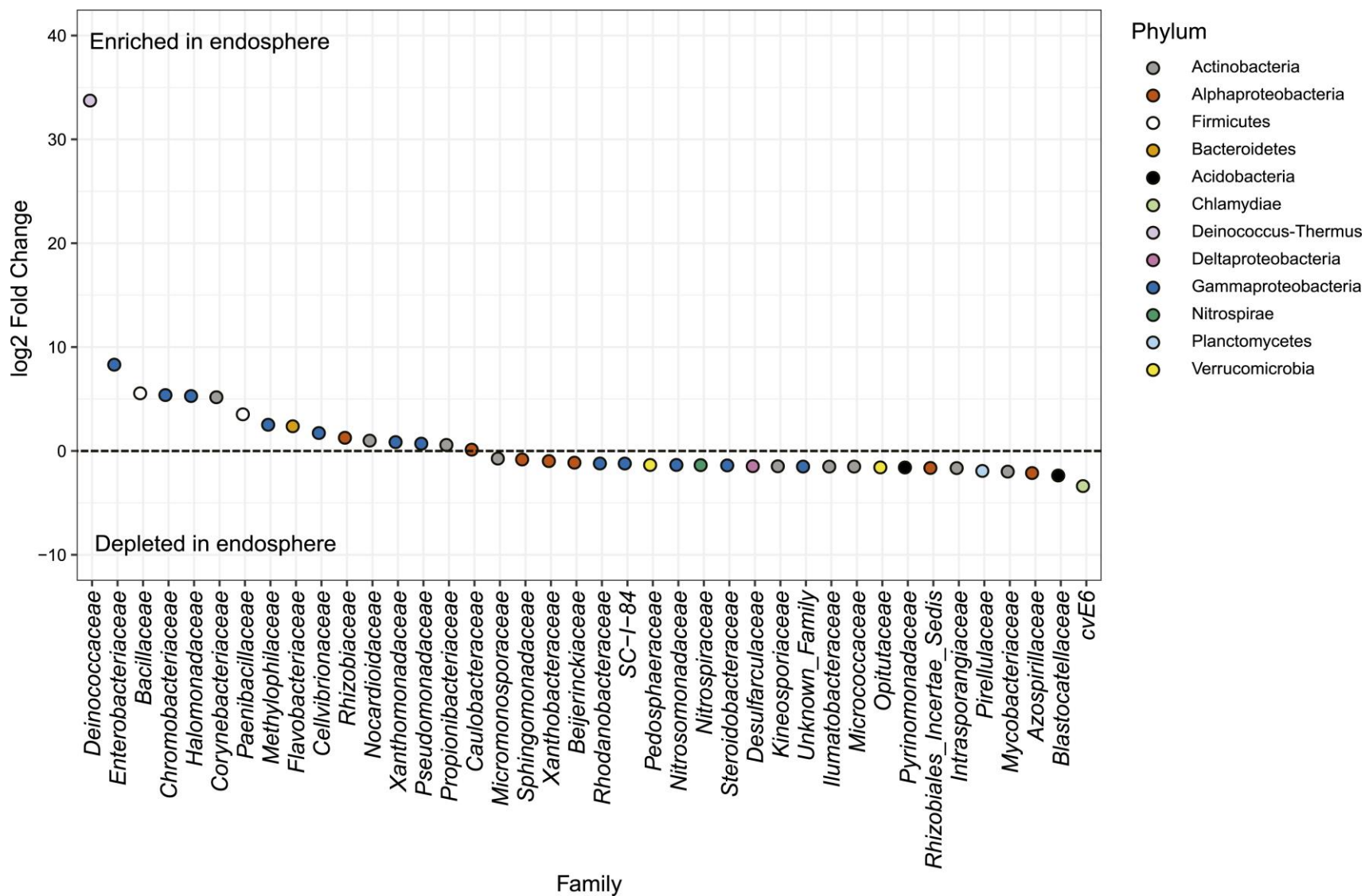


Figure 3.2. Log2 fold changes of significantly enriched or depleted families in the rhizosphere to endosphere compartments (genotype independent). Each point represents one family, colored by phylum. Points above the dotted line represent families significantly enriched in the endosphere compared with the rhizosphere, and points below represent depleted families.

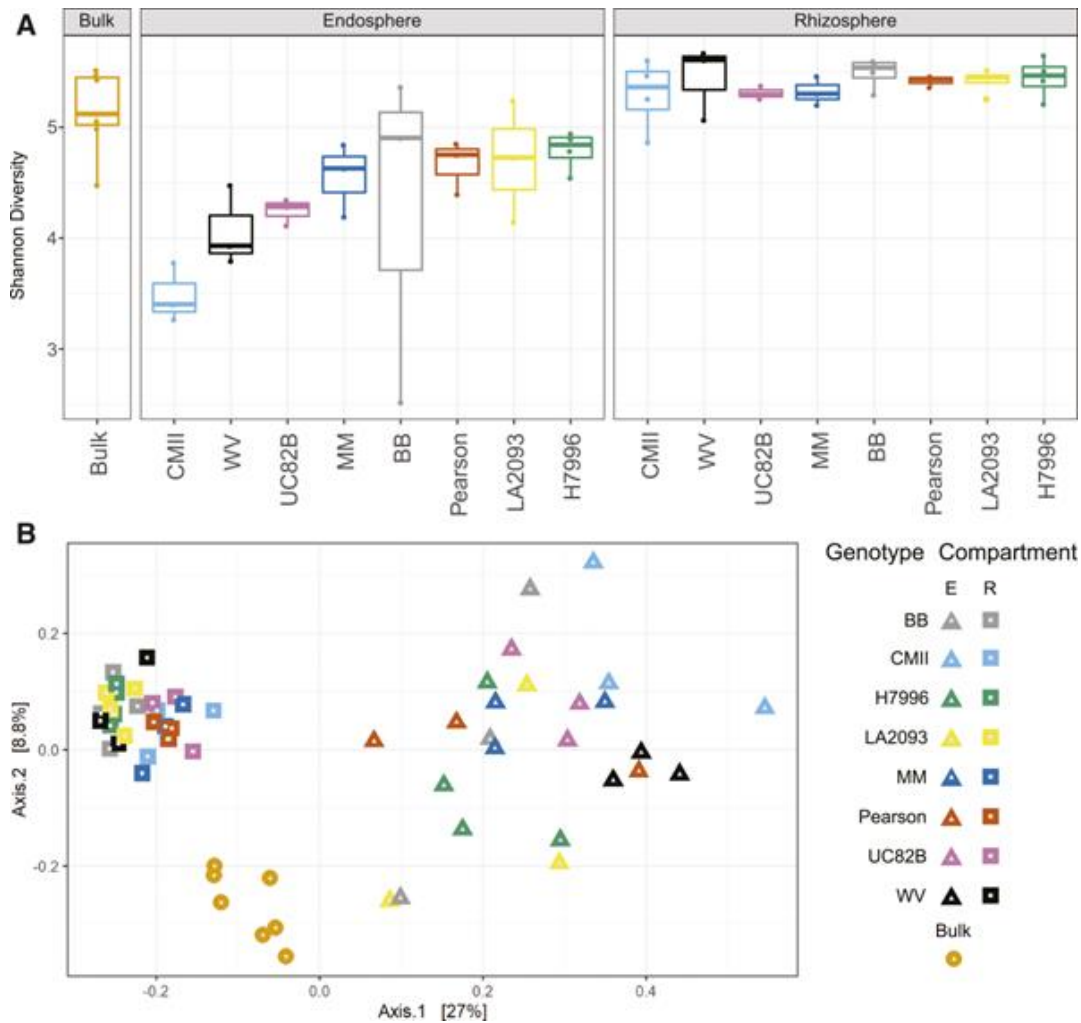


Figure 3.3. Root endosphere α and β diversity varies by compartment and genotype. A, Boxplot of Shannon diversity of rhizospheres and endospheres of all eight genotypes and bulk (unplanted) soil. B, Principle coordinate analysis of Bray-Curtis distance among bacterial communities of all eight genotypes. Squares indicate rhizosphere samples, triangles indicate endosphere samples, and orange circles indicate bulk samples. Colors indicate genotype as labeled in the legend. Abbreviations: E = endosphere, R = rhizosphere, CMII = *Solanum lycopersicum* ‘Castlemart II’, WV = *S. pimpinellifolium* accession WestVirginia700, UC82B = *S. lycopersicum* ‘UC82B’, MM = *S. lycopersicum* ‘Money Maker’, BB = *S. lycopersicum* ‘Bonnie Best’, Pearson = *S. lycopersicum* ‘Pearson’, LA2093 = *S. pimpinellifolium* accession LA2093, and H7996 = *S. lycopersicum* ‘Hawaii7996’.

We next asked whether the host genotype contributed to variation in β -diversity in either the rhizosphere or endosphere. We separated the dataset and focused on the endosphere and rhizosphere compartments separately, and subsequently used CAP analysis to quantify the effect of tomato genotype on microbial diversity within each root microhabitat. In the rhizosphere, genotype explained 34.8% of the variation (permutest, 1000 permutations, Genotype: $F_{7,27} = 1.53$, $p < 0.002$) (Figure 3.4A). The clustering pattern revealed two groups: one consisting of the *S.*

lycopersicum genotypes H7996, BB and the *S. pimpinellifolium* WV and LA2093; the other was composed of all other *S. lycopersicum* genotypes. The reason for such a clustering pattern is not clear. In the endosphere, genotype explained 32.8% of the variation (permutest, 1000 permutations, Genotype: $F_{7,24} = 1.185$, $p = 0.037$) (Figure 3.4B). In the endosphere, replicates of individual genotypes tended to cluster together, although there was some overlap among genotypes.

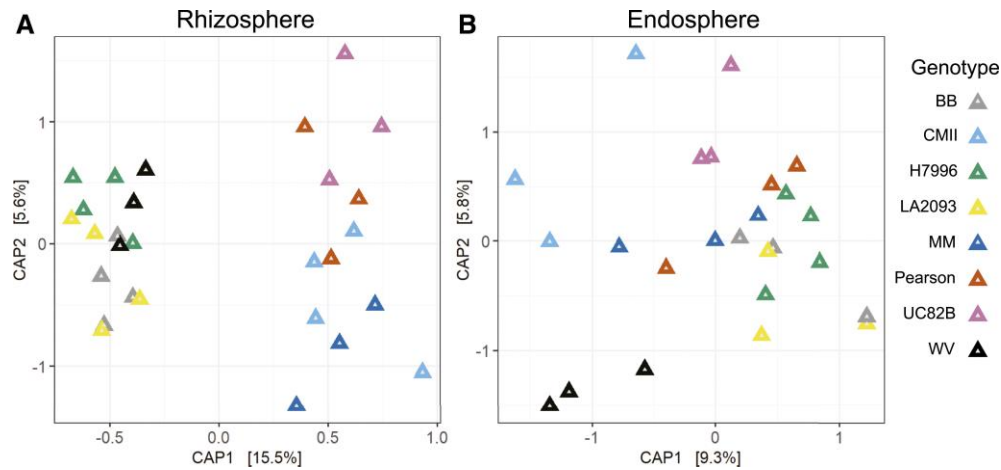


Figure 3.4. Genotype plays a significant role in shaping both rhizosphere and bacterial endosphere communities. Canonical analysis of principle coordinates (CAP) of the A, rhizosphere and B, endosphere with genotype as a constraining factor. Abbreviations: E = endosphere, R = rhizosphere, CMII = *Solanum lycopersicum* ‘Castlemart II’, WV = *S. pimpinellifolium* accession WestVirginia700, UC82B = *S. lycopersicum* ‘UC82B’, MM = *S. lycopersicum* ‘Money Maker’, BB = *S. lycopersicum* ‘Bonnie Best’, Pearson = *S. lycopersicum* ‘Pearson’, LA2093 = *S. pimpinellifolium* accession LA2093, and H7996 = *S. lycopersicum* ‘Hawaii7996’.

3.4.3 Tomato genotypic differences in family level abundances in the root endosphere

The characterization of microbiome β -diversity revealed an important contribution of tomato genotype to the root bacterial microbiome. To further explore the role of tomato genotype, we investigated whether any taxa were enriched in the root endosphere all genotypes. We identified two bacterial families enriched in the endosphere of at least six of eight tomato genotypes. These families were called ‘high-frequency endosphere colonizers’ (Figure 3.5A). High-frequency endosphere colonizing families included *Bacillaceae*, composed of 6 ASVs (Figure 3.5A), and *Rhizobiaceae*, with 26 ASVs (Figure 3.5C). *Bacillaceae* was enriched in the endosphere of all eight genotypes while *Rhizobiaceae* was enriched in all genotypes except UC82B and Pearson. The abundance of high frequency endosphere colonizers varied quantitatively across the eight tomato genotypes (Figure 3.5A, C). *Bacillaceae* abundance in the endosphere ranged from 3.6% in BB to 13.3% in CMII. *Rhizobiaceae* abundance ranged from 2.4% in UC82B to 6.7%

in CMII. Of the high-frequency endosphere colonizers, the most highly abundant ASV across all genotypes was ASV2, a *Bacillaceae* in the genus *Anaerobacillus*. The relative abundance (proportion of reads for each family out of the total number of reads in each sample) of all families in the endosphere and rhizosphere for each genotype is shown in Appendix Figure B3.3 and Appendix Table B3.18.

To investigate selection of these high frequency endosphere colonizers by different tomato genotypes, we examined their abundance in the root endospheres of 20 RILs from a cross of *S. lycopersicum* H7996 and *S. pimpinellifolium* WV. As with other tomato genotypes, the RILs showed highest abundance of Proteobacteria, followed by Actinobacteria, Firmicutes, and Bacteroidetes (Appendix Figure B3.4). Although the RIL parent genotypes, WV and H7996, have significantly different α -diversity in the endosphere by t -test, RIL α -diversity in the endosphere showed no significant differences among genotypes (ANOVA, Genotype: $F_{23,86} = 1.284$, $p = 0.216$) (Appendix Figure B3.5A). β -diversity was not significantly different among RILs but showed trends towards genotypic differences (PERMANOVA, Genotype: $F_{23,86} = 1.102$, $p = 0.104$) (Appendix Figure B3.5B).

Because differences in root architecture traits have been correlated with different root microbiota (Perez-Jaramillo et al. 2017), we examined whether there was a correlation between these traits and relative abundance of high frequency colonizers as well as overall α -diversity. No relationship was found between root architecture traits and any root microbiota measures tested (Appendix Figure B3.6A and B, data shown for total root length). Plant resistance to pathogens has also previously been correlated with different root microbiota. The parents of the RIL population, H7996 and WV, are resistant and susceptible, respectively, to the soil borne bacterial pathogen *Ralstonia solanacearum*, and our RILs segregate for resistance (Appendix Table B3.2). As with root architecture, we did not find a relationship between levels of immunity to *R. solanacearum* and the high-frequency colonizer families or overall α -diversity (Appendix Figure B3.6C and D).

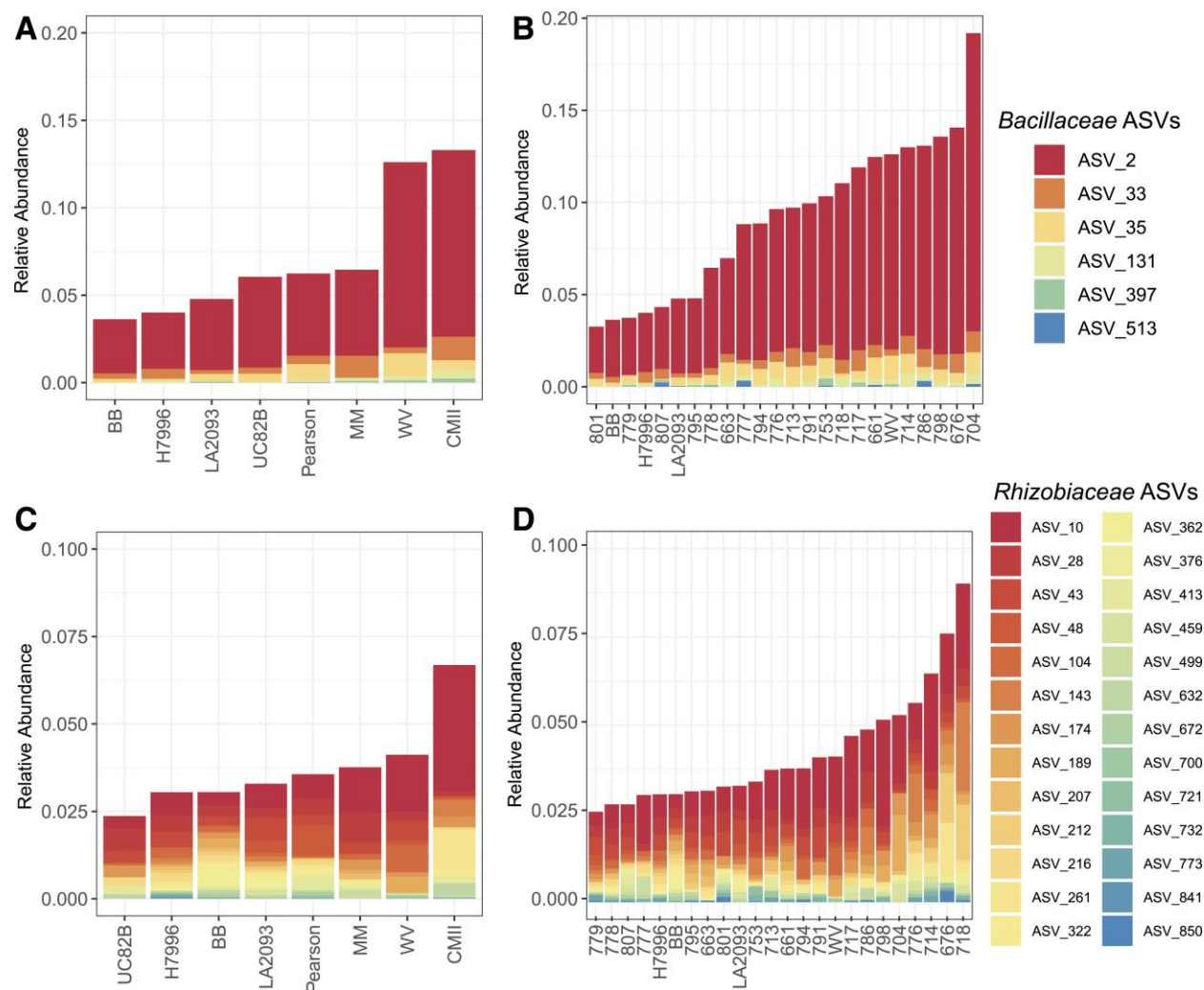


Figure 3.5. High-frequency colonizer families vary quantitatively in abundance across genotypes. Abbreviations: BB = *Solanum lycopersicum* ‘Bonnie Best’, H7996 = *S. lycopersicum* ‘Hawaii7996’, LA2093 = *S. pimpinellifolium* accession LA2093, UC82B = *S. lycopersicum* ‘UC82B’, Pearson = *S. lycopersicum* ‘Pearson’, MM = *S. lycopersicum* ‘Money Maker’, WV = *S. pimpinellifolium* accession WestVirginia700, and CMII = *Solanum lycopersicum* ‘Castlemart II’. Relative abundance of *Bacillaceae* across A, all eight genotypes and B, 20 recombinant inbred lines (RILs) derived from H7996 and WV. Relative abundance of *Rhizobiaceae* across C, all eight genotypes and D, RILs derived from H7996 and WV. Numbered genotypes indicate RILs. BB and LA2093 are included to show variation due to species. Different colors represent individual amplicon sequence variants (ASVs) represented in each family.

Examination of the two families in the high-frequency endosphere colonizer groups showed variation in the abundance of these families across the 20 RILs (Figure 3.5B: *Bacillaceae*, 3.5D: *Rhizobiaceae*). ASV 2 (Genus: *Anaerobacillus*) showed the highest abundance in the RIL root endospheres, similar to the previously examined genotypes. RILs showed transgressive segregation (i.e. phenotypes more extreme than either parent) for the abundance of frequent colonizers. For example, ASV2 was found to be ~4% of the root endosphere in the H7996 parent and ~9.5% of the root endosphere of WV, but varied from 2% in RIL 801 to ~14% in RIL 704.

3.4.4 A *Bacillaceae* isolate differentially promotes growth across a set of RILs

We hypothesized that high-frequency endosphere colonizing taxa would be beneficial for tomato growth, but this growth effect may differ by genotype. To examine this further, we first searched for a bacterial isolate from tomato roots that we could cultivate and was in the same family as the high-frequency colonizers. We isolated root endophytes from roots of six field-grown tomato plants: three H7996 and three WV. Plants were grown in the spring and summer of 2017 in the same farm from which soil was harvested for the microbiome greenhouse experiments described above.

We isolated 183 bacterial colonies from the inner root tissues of the six plants. Using Sanger sequencing of the 16S rDNA gene we identified an isolate (HA129) in the *Bacillaceae* family that was 97.1% identical to ASV397. Although HA129 was isolated from roots of H7996, it was also 99.5% identical to an isolate from WV. To test whether tomato genotypes exhibited variation in their response to *Bacillaceae*, we tested the response of 16 RILs plus the parents H7996 and WV to the isolate HA129. Ten days after germination, nine replicates of each RIL, H7996, and WV were inoculated with HA129 and nine with sterile 1 X PBS (pH = 7.4) as a control. After 24 days, plants were harvested. Fresh weight of roots and shoots were compared to that of mock-inoculated controls. RILs and parental lines significantly differed in their response to HA129 for both root and shoot growth (Figure 3.6). Two RILs and WV showed less than 5% growth promotion in response to HA129 compared to mock-inoculated controls. In contrast, H7996 and three RILs showed more than 30% shoot growth promotion. Response rates for roots and shoots were similar to each other across the RILs. Although RILs varied in their growth response to HA129, there was no relationship (adjusted $R^2 = 0.05123$) between the growth response rate of an

RIL to the *Bacillaceae* HA129 and the relative abundance of *Bacillaceae* in an RIL's root endosphere in the microbiome experiment.

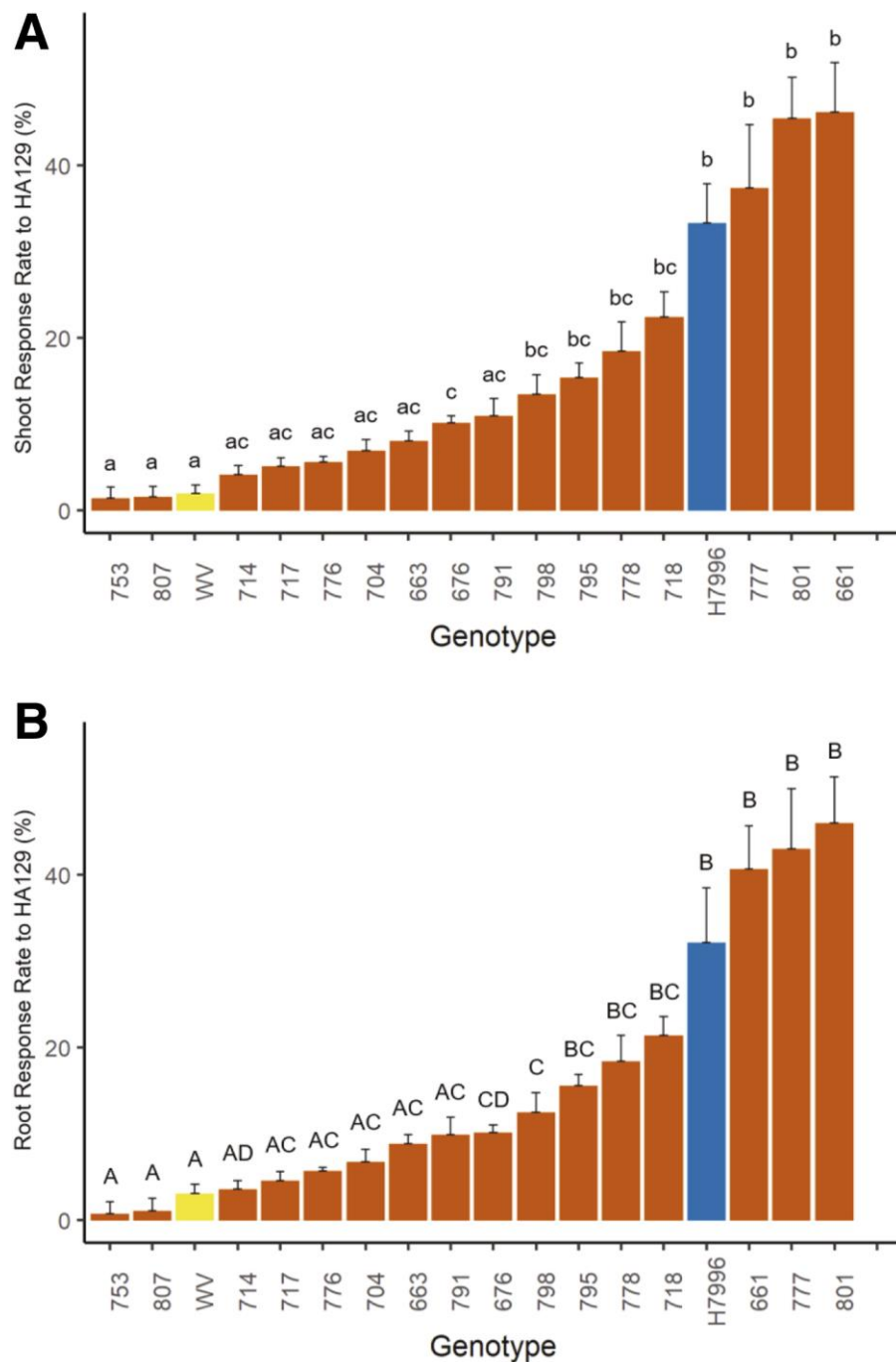


Figure 3.6. Recombinant inbred lines (RILs) show quantitative differences in their response to *Bacillaceae* isolate HA129. Response rate was measured in terms of the change in A, shoot or B, root weight of inoculated RILs compared with mock-treated plants of the same genotype. Bar plot showing response rate of different genotypes is color-coded in orange (RILs), blue (parent *Solanum lycopersicum* 'Hawaii7996' [H7996]), and yellow (parent *S. pimpinellifolium* accession WestVirginia700 [WV]) (n = 9 per genotype; different letters indicate significantly different at P < 0.05).

3.5 Discussion

3.5.1 Tomato genotypes have distinct but overlapping root bacterial microbiomes

We show that genetic variation in cultivated and wild tomatoes impacts selection of the root bacterial microbiome, and that tomato genotypes differentially respond to a bacterial endophyte. We find that within *S. lycopersicum* and *S. pimpinellifolium*, tomato genotype significantly impacted microbial community diversity in both the root endosphere and rhizosphere. *Bacillaceae* and *Rhizobiaceae* were enriched in the endospheres of at least six of the eight genotypes and varied quantitatively in abundance among a set of RILs. Inoculation of 16 RILs and their parents with an isolate in the same family as the high-frequency colonizers revealed that this isolate promoted both root and shoot growth in a genotype-dependent manner. Together, these data suggest a genetic underpinning to tomato selection and responses to root microbiota and that microbiome strategies to improve tomato production should consider the specific tomato cultivar utilized.

The tomato rhizosphere displayed a rhizosphere effect, with significantly different bacterial communities compared to bulk soil. Proteobacteria and Actinobacteria, with smaller abundances of Bacteroidetes, composed a significant portion of the tomato root rhizosphere microbiota. These phyla were also present in the rhizosphere of barley (Bulgarelli et al. 2015), Arabidopsis (Bulgarelli et al. 2012; Lebeis et al. 2015), maize (Peiffer et al. 2013, Fitzpatrick et al. 2018) and in other tomato rhizosphere microbiomes (Poudel et al. 2019; Lee et al. 2019; Cheng et al. 2020). Similar to tomato rootstocks (Poudel et al. 2019), Acidobacteria were not abundant in our study of tomato root rhizospheres. However, Acidobacteria were abundant in rhizospheres of tomatoes grown in Chinese and South Korean soils, so this difference may be location or soil-dependent (Lee et al. 2019; Cheng et al. 2020).

The tomato root endosphere microbiota was dominated by Proteobacteria, Actinobacteria and Firmicutes, with lower levels of Bacteroidetes. Firmicutes were enriched in the endosphere compared to the rhizosphere. This is in contrast to the bacterial communities of grafted tomato root stocks (Poudel et al. 2019). The difference could have been due to soil or age of plants, or due to differences in the type of root examined. We sampled whole roots of seedlings, while Poudel et al harvested small lateral roots at the time of tomato harvest. Different types of roots can have significant differences in their microbial communities (Kawasaki et al. 2016).

Consistent with other root bacterial microbiome studies, distinct microbial communities exist in each tomato root compartment, with a decrease in microbial diversity from rhizosphere to endosphere (Lebeis et al. 2015; Bulgarelli et al. 2015; Edwards et al. 2015; Fitzpatrick et al. 2018; Poudel et al 2019; Caradonia et al. 2019; Lee et al. 2019). Differences between compartments explained 30% of the variation in tomato root bacterial communities, very similar to those of tomato rootstocks (Poudel et al. 2019), and field-grown tomato roots treated with different nitrogen fertilizers (Caradonia et al. 2019).

3.5.2 Tomato genotype contributes to both rhizosphere and endosphere microbiome assembly

The rhizosphere and root endosphere are distinct microenvironments that each exert unique pressures on microbiota. We identified a compartment x genotype interaction, and when compartments were examined separately, CAP analysis revealed that genotype contributed over 32% of the variation in rhizosphere samples, and over 33% to the variation among endosphere samples. Within compartments, the impact of genotype appears to differ according to species. In a study of six rice cultivars, the rhizosphere had a greater genotypic effect on the root bacterial communities compared to the endosphere (30% compared to over 12%; Edwards et al. 2015), while among 36 olive cultivars, genotype explained 42% of the variation in the endosphere and over 53% in the rhizosphere (Fernandez-Gonzalez et al. 2019).

Previous studies have shown that more genetically dissimilar hosts have more dissimilar root bacterial microbiomes (Bouffaud et al. 2014; Naylor et al. 2017). The root bacterial microbiomes of wild species tend to be distinct from those of their domesticated relatives (Shenton et al 2016; Bulgarelli et al. 2015; Chaluvadi and Bennetzen 2018). We examined both fresh market and processing cultivated tomatoes (*S. lycopersicum*) and *S. pimpinellifolium*, the closest wild relative to *S. lycopersicum*. We did not identify clustering patterns in beta diversity based on the distinction between market and processing tomatoes, nor on wild versus domesticated species. Our CAP analysis revealed a clustering pattern in the rhizosphere that was unrelated to species, and no clear clustering in the root endosphere. The lack of distinction between our wild and domesticated species could be because they are close relatives.

Although we found that tomato genotype contributed over 30% of the variation in each compartment, there was greater dispersion among endosphere samples from different genotypes

compared to those of the rhizosphere. This has previously been observed among cultivars of many other species, including grapevine rootstocks (Marasco et al. 2018), olive (Fernandez-Gonzalez et al. 2019), poplar (Beckers et al. 2017), and cotton (Wei et al. 2019), among others. Models of microbiome assembly posit that the root endosphere acts as a gate which allows only a subset of rhizosphere and rhizoplane microbes to enter inner root tissues (Edwards et al. 2015; Bulgarelli et al. 2013). The dissimilarity among endosphere microbiomes of different genotypes could suggest that such a gate operates differently in distinct genotypes. The inside of the root exerts unique pressures on bacteria, which may contribute to the decreased species diversity of this compartment and result in greater dispersion among genotypes. Endophytes must be able to suppress the plant immune system (Liu et al., 2017; Yu et al. 2019), and directly withstand host metabolites, nutrients, and signaling molecules, all of which may vary by genotype. For example, a screen of diverse tomato accessions revealed extensive natural variation in their responses to bacterial MAMPs like flg22 and flgII-28 (Roberts et al. 2019; Veluchamy et al. 2014). Thus, root endophytic bacteria may differentially repress host immune responses depending on host genotype. Such direct and distinct genetic pressures could result in the greater dispersion among endosphere bacterial microbiomes of different tomato genotypes.

Tomato rhizosphere communities of different genotypes also differed from each other, though to a lesser extent compared to those of the endosphere. In the rhizosphere, chemical cross-talk, mediated largely through root exudation and microbial activity, is critical for root-microbe interactions. Root exudates include a large and diverse range of primary and secondary metabolites that mediate rhizosphere microbiome structure and activity in tomato and other species (Sasse et al. 2018; Rajniak et al. 2018; Stringlis et al. 2018; Zhelnina et al. 2018). Root exudate secretion varies by soil environment, root developmental stage, and plant species (Sasse et al. 2018; Badri and Vivanco 2009). Differences among genotypes in their root exudation profiles, root cell release, and root mucilage production likely lead to differences in rhizosphere microbiota among genotypes, and may account for the genotype effect we observed in the tomato rhizosphere.

The similarity of rhizosphere samples among our genotypes relative to the endosphere may be a result of the young age of the plants used in our study. Plant age is an important determinant of the rhizosphere microbiome (Lundberg et al 2012; Chaparro et al 2014; Edwards et al. 2018; Walters et al 2018; Hu et al 2020) and may result from changes in root exudation over the course of plant development (Chaparro et al 2014). It is possible that at such an early stage of development,

tomato root rhizodeposits may not exert a heavy selection pressure on the rhizosphere, while the root endosphere exerts a heavier pressure on potential endophytic colonizers. Consistent with our data showing small, but distinct community differences between the bulk and rhizosphere soil at an early vegetative stage, a recent study examining temporal variation in the tomato rhizosphere microbiome showed increasing differences between bulk soil and rhizosphere samples with plant developmental stage (Hu et al 2020). Further examination of compartment x plant age effects on the root microbiome will be important to further understand how roots recruit and gate their microbial communities.

3.5.3 The potential for tomato genotype to select for root microbiota

One agricultural microbiome management strategy is to design inoculants consisting of microbial consortia optimized for specific plant genotypes. This assumes that host-selected microbes have a beneficial effect on the host plant, and that host selection and responses are genetically encoded with sufficient heritability to make breeding effective.

Several studies, including this work, suggest that this is possible. In a large study of the rhizosphere microbiota of 27 maize inbred lines and ~ 4800 samples across three fields, the abundance of nearly 150 OTUs was significantly regulated by genotype (Walters et al. 2018). Heritability for these microbes was low, and it is not known whether these taxa provide beneficial functions. Here, we found that multiple tomato genotypes recruit members of the families *Bacillaceae* and *Rhizobiaceae*, and that a panel of related tomato genotypes varied in growth responses to a *Bacillaceae* isolate identified from the tomato root endosphere. Variation in growth responses among these genotypes may indicate an element of genetic control, although additional experiments with larger populations are necessary. Genetic control was also postulated in a previous study that identified three tomato QTL for biocontrol of *Bacillus cereus* UW85 to the tomato root rot pathogen *Pythium torulosum* (Smith et al. 1999).

Notably, our work was performed under non-stress conditions. Additional work is necessary to understand the impact of environment. Given the range of variation in abiotic stress responses in cultivars, particularly drought stress responses, one may hypothesize that the impact of genotype on root bacterial communities would be greater under stress. However, Naylor et al. 2017, found that among 18 grass species, the root endosphere bacterial microbiota became more similar in drought compared to plants grown in well-watered conditions. Perhaps in some

environments the impact of the soil environment overrides that of genotype. In such cases breeding for genotype-specific host-microbiome relationships may be less important than identifying environment-specific microbiomes.

The success of using genotype-specific microbiomes in agriculture will also depend on which plant traits significantly impact root microbiota, and how much we can manipulate such traits without deleteriously impacting other aspects of plant growth and development. Although root bacterial microbiome communities may impact root architecture (Perez-Jaramillo et al. 2017), the degree to which plants with distinct types of roots or root architectures select for specific taxa remains uncertain. We did not find a relationship between the root endosphere microbiome and root architecture in our tomato RILs, suggesting that at least in this population, selecting for specific root architecture traits would not select for specific taxa. Similarly, we did not observe a relationship between microbiota and immune responses. The RILs we used were derived from genotypes that are resistant (H7996) and susceptible (WV) to a soilborne bacterial pathogen, *R. solanacearum*. We did not identify a relationship between specific taxa or α -diversity of RIL root endosphere microbiomes with the level of RIL resistance, although this could be because our soils were not infected with *R. solanacearum*. Additional work is needed to understand how plant traits such as root architecture and disease resistance impact selection of microbial communities.

Finally, another important aspect when considering the role of host genotype is the importance of understanding the genetics underlying both host selection and responses. We did not see a relationship between genotypic variation in relative abundance of *Bacillaceae* and growth response to inoculating with a *Bacillaceae* isolate. This may be because isolate HA129 is not identical to any of the *Bacillaceae* identified in the greenhouse microbiome experiment, or because other factors besides isolate abundance contribute to growth promotion. Alternatively, we know little about the relationship between colonization levels of root associated microbiota and plant growth responses, nor whether this relationship varies across genotypes. Perhaps different cultivars require less bacterial colonization for similar growth responses. While we did not measure colonization levels of HA129 here, future work aiming to understand the relationship between recruitment, bacterial colonization and plant growth responses is necessary.

3.6 Conclusions

Our results reveal the structure of the tomato root bacterial microbiome, and show that tomato genotype impacts the selection of both rhizosphere and endosphere bacterial taxa. These data suggest that taxa present in the microbiome of multiple tomato genotypes promote growth, and hint at a genetic basis for response to root endophytic bacterial taxa. This suggests that breeding tomatoes for improved associations with root microbial communities is possible, although future work with RIL or GWAS tomato populations is needed to investigate this fully.

Many aspects of genotype-microbiome interactions remain to be understood if we are to use microbiomes to benefit crop production. For example, the effect of genotype varies among species, plant developmental stage, root compartment, and abiotic stress. Interactions among these add additional complexity, and how much one could rely on breeding as a strategy for optimal microbiome use is unclear. Because plants likely select for specific microbial functions, regardless of taxa, additional studies examining the role of host genotype in selecting microbial function are needed. Future work is also needed to understand the heritability of QTL which select for the microbiome, and how such QTL could be used as part of a multi-pronged microbiome management approach. For example, combining these QTL with field inoculation of taxa selected by the host may improve production. Although much work is still needed, our work suggests that optimizing host genetics may be one way to select for beneficial microbiomes in tomato.

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CHAPTER 4. BENEFICIAL BACTERIA INDUCE CHANGES IN PHENOTYPES OF SPECIFIC ROOT CELLS DURING EARLY INTERACTION

4.1 Abstract

Root-associated bacteria gain enormous potential for use in sustainable agricultural management. However, the application of microbial products has not yielded satisfying results in the field, partially due to the limited understanding of symbiotic interaction between the root and its microbes. Utilizing the tomato microbiome system, we investigated whether beneficial bacteria induce changes in specific host cell types to promote plant growth. From our bacterial endophyte collection, we identified two isolates that dramatically stimulate the root length of seedlings of the domesticated tomato genotype H7996 (*Solanum lycopersicum*). Cross-sectioning of root tissues suggested the two isolates induced cell-type specific response in inoculated roots with a significant increase of cell wall lignification in the vasculature. Interestingly, the impact of the two isolates on root phenotypes was only observed on H7996, but not in its original host, the wild tomato species, WV (*S. pimpinelifolium*). Although both tested strains were isolated in the root endosphere, one might be more efficient in colonizing the root interior than another on the root of H7996 seedlings. However, both strains persistently presented on the root rhizoplane. Changes in root cell phenotype were also independent of the initial concentration of the bacterial inoculum. Additional studies are needed to uncover a possible correlation between the observed vasculature lignification and the growth-promoting effect of beneficial bacteria on plants. Knowledge of cell-type specific responses is critical for developing effective strategies for enhanced host associations with soil microbes while mitigating host susceptibility to pathogens.

4.2 Hypothesis (Developing project)

Plant-growth-promoting bacterial endophytes induce changes in root cell phenotypes to establish mutualistic interaction.

4.3 Results

4.3.1 Two bacterial endophytes, WV180 and WV182, stimulated the root growth of H7996 seedlings at the highest rate compared to other tested isolates.

To identify plant-growth-promoting bacteria that lead to changes in root cell phenotype, tissues of mock- and bacterial-inoculated roots were hand-sectioned and examined with the epifluorescence microscope. Five-day-old seedlings of the two genotypes H7996 (*Solanum lycopersicum*) and WV (*S. pimpinellifolium*) growing on 1% agar plates were individually inoculated with six bacterial isolates, HA22, HA28, WW44, HA141, WV180, and WV182. These isolates were selected from our tomato bacterial endophyte collection described in Tran et al. 2022. Sanger sequencing of the 16S rRNA gene identified all six isolates were members of *Pseudomonas* spp (Tran, French, and Iyer-Pascuzzi 2022). Isolate WW44 promoted the growth of H7996 seedlings in the greenhouse, while seedlings inoculated with isolate HA22 and WV182 did not impact plant growth in greenhouse conditions (Tran et al. 2022). The two isolates HA141 and HA28 expressed intermediate growth phenotypes on inoculated seedlings, which was statistically insignificant in both the growth-promoting and non-growth-promoting groups (Tran, French, and Iyer-Pascuzzi 2022). Isolate WV180 was identified as a member of the plant-growth-promoting group Siderophore from the in vitro experiment, but was not selected to test its impact on plant growth in the greenhouse (Tran et al. 2022).

From the six isolates selected for the in vitro plant-binary interaction experiment, we identified two isolates, WV180 and WV182, that promoted root growth of H7996 seedlings at the highest rates when compared to others at 72-hour-post-inoculation (hpi). Bacterial impact on root length was calculated by the effect size, measured by Cohen's *d*, of inoculated roots in relation to mock samples. In H7996 seedlings, the length of WV180- and WV182-inoculated roots improved by 9.43 ± 0.077 and 5.66 ± 0.055 cm, respectively (Figure 4.1A). Although both isolates were originally recovered from the root endosphere of the field-grown WV, the two bacteria did not stimulate the root growth of their original host, WV, at the same levels as in H7996 (Figure 4.1B). At 72 hpi, isolate WV180 on H7996 stimulated root growth to more than 4cm (9.43 ± 0.077 cm change in root length) when compared to its effect on WV (5.09 ± 0.049 cm). Roots of WV inoculated with isolate WV182 increased an additional growth of 3.26 ± 0.052 cm, but 5.66 ± 0.055 cm on H7996 at 72 hpi.

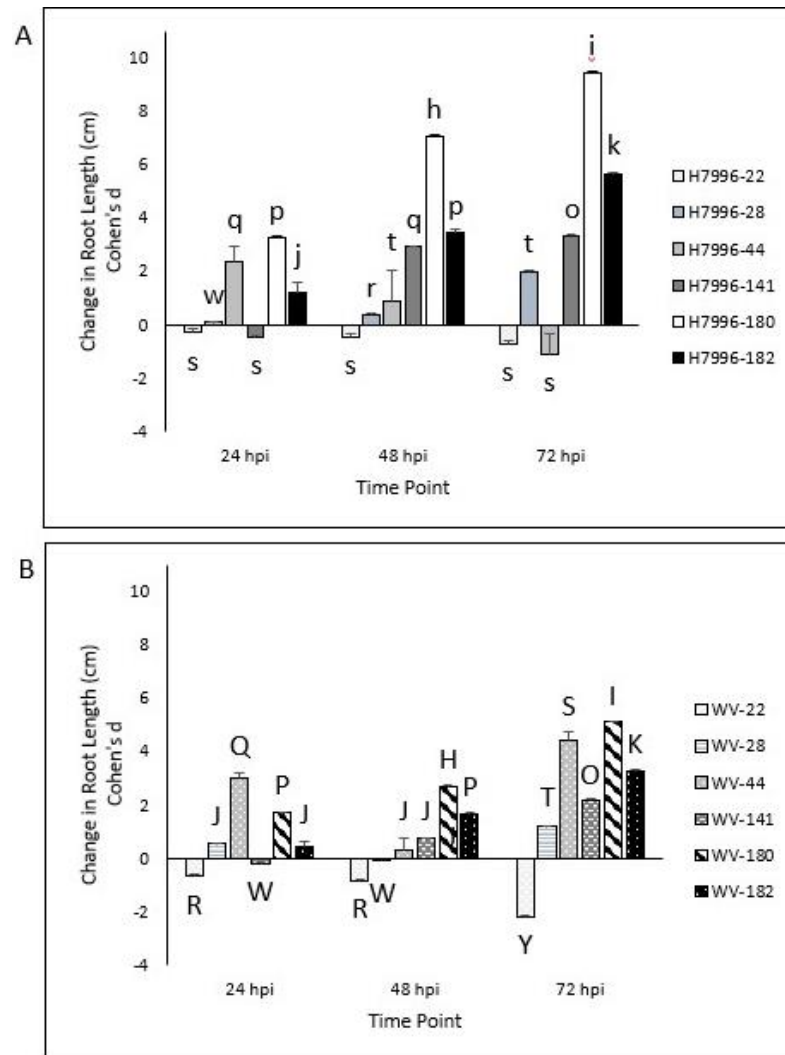


Figure 4.1. Isolates WV180 and WV182 significantly induce root elongation in tomato seedlings of (A) H7996 and (B) WV. Bar plot describes the change in root length when inoculated with selected isolates calculated as the effect size Cohen's d between the bacterial-treated and mock-treated groups. n=6 plants per isolate; different letters indicate significantly different at $P < 0.05$ from Tukey's posthoc test. H7996, *S. lycopersicum* cv. Hawaii7996. WV, *S. pimpinellifolium* accession WestVirginia700. 22, isolate HA22. 28, isolate HA28. 44, isolate WW44, 141, isolate HA141. 180, isolate WV180. 182, isolate WV182.

4.3.2 The endophytes might not need to colonize the root endosphere at a high level to express beneficial effect.

We asked whether the two isolates needed to internalize into the root endosphere compartment to promote root growth of H7996. We examined the root colonization progress of the two isolates on five-day-old H7996 seedlings at 96, 120, 144 hpi. Colonization experiments suggested that both isolates colonized the root rhizoplane (root surface) of H7996 in high density, ranging between 3.5 and 4.5 log of bacterial load per gram of fresh root tissue for both isolates at all three observed time points (Figure 4.2A). However, isolate WV180 had a low

colonization level in the root endosphere of H7996, under 1 log of bacterial load per gram of fresh root tissue at 96 hpi and also failed to thrive at later time points (Figure 4.2B). In contrast, isolate WV182 colonized the root endosphere of H7996 at a much higher rate, between 2.5 and 3 log of bacterial load per gram of fresh root at 96, 120, and 144 hpi.

Bacterial load in the endosphere of both isolates were lower than on the rhizoplane, the root's surface, at all time points. Interestingly, Root elongation stimulated by isolate WV180 is more profound than isolate WV182 even when its colonization in both root compartments was not as high (Figure 4.1A). There are two possible explanations for this observation: (1) the tested isolates might not need to internalize inside the root to stimulate its growth, or (2) the colonization rate in the endosphere is not dependent on the effect of the two isolates on promoting root growth.

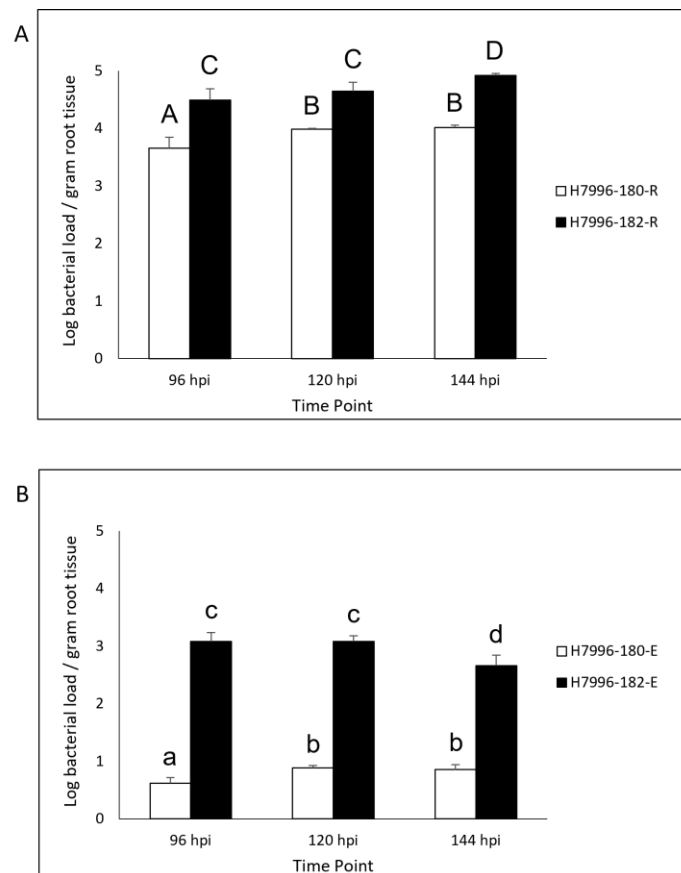


Figure 4.2. Isolates WV180 and WV182 colonize at different levels in (A) the root rhizoplane and (B) the root rhizosphere of H7996 seedlings. Barplot describes bacterial colonization rate of the two isolates WV180 and WV182 at 96-, 120-, and 144-hpi. n=6 plants per isolate; different letters indicate significantly different at $P < 0.05$ from Tukey's posthoc test. H7996, *S. lycopersicum* cv. Hawaii7996. 180, isolate WV180. 182, isolate WV182.

4.3.3 Plants inoculated with beneficial root endophytes show increased the number of fluorescent cells in the root vasculature.

We next asked whether the two root-growth-promoting isolates, HA180 and HA182, induce physiological changes in inoculated H7996 roots. We examined cross-sections of inoculated roots at 96-, 120-, and 144-hour-post-inoculation (hpi). At the designated time point, 1cm of fresh root tissues at 2.5cm under the root-shoot junction were processed and observed under the epifluorescence microscope. Compared to the mock-treated samples, UV illumination showed a significant increase in autofluorescent of the cells in the vasculature in inoculated roots compared to mock-treated samples (Figure 4.3). The isolate WV-182 might promote more xylem vessels of H7996 seedlings to be fluoresced compared to the isolate WV180 ($P < 0.05$). Because the isolate WV182 colonized the root in higher levels in both root compartments and induced a more vigorous cell-type specific response in the inoculated host compared to WV180, we asked whether the increased number of fluorescent cells in the xylem were dependent on the inoculum concentration. Inoculation experiments of the isolate WV182 at three different concentrations (10^3 , 10^5 , and 10^7 cfu/mL) suggested the increased number of fluorescent xylem cells in roots of H7996 at 144hpi was not influenced by the initial inoculum concentration of WV182. These results suggested structural changes in seedling roots in response to the presence of beneficial microbes are not influenced by the density of the beneficial microbes in the surrounding environment.

The autofluorescence of xylem vessels might indicate the induction of phenolic compounds, most typically lignin, constituting the cell wall structure (Novo et al. 2017; Zeiss et al. 2019; Kashyap et al. 2021). Phloroglucinol-HCl staining suggested both isolates WV180 and WV 182 induced lignification of the xylem vessels of inoculated roots (Figure 4.3B). Autofluorescence in the xylem of the phloroglucinol-HCl stained samples was not quenched under UV illumination (Figure. 4.3B). This observation suggested other phenolic compounds, in addition to lignin, were induced in roots during interaction with WV180 and WV 182. Cell wall lignification has been documented as an inducible physical barrier as host defense against pathogens (Jhu et al. 2022; Joo et al. 2021; Lee et al. 2019; Kashyap et al. 2022). However, the role of cell wall lignification in beneficial plant-microbe interaction remains to be investigated.

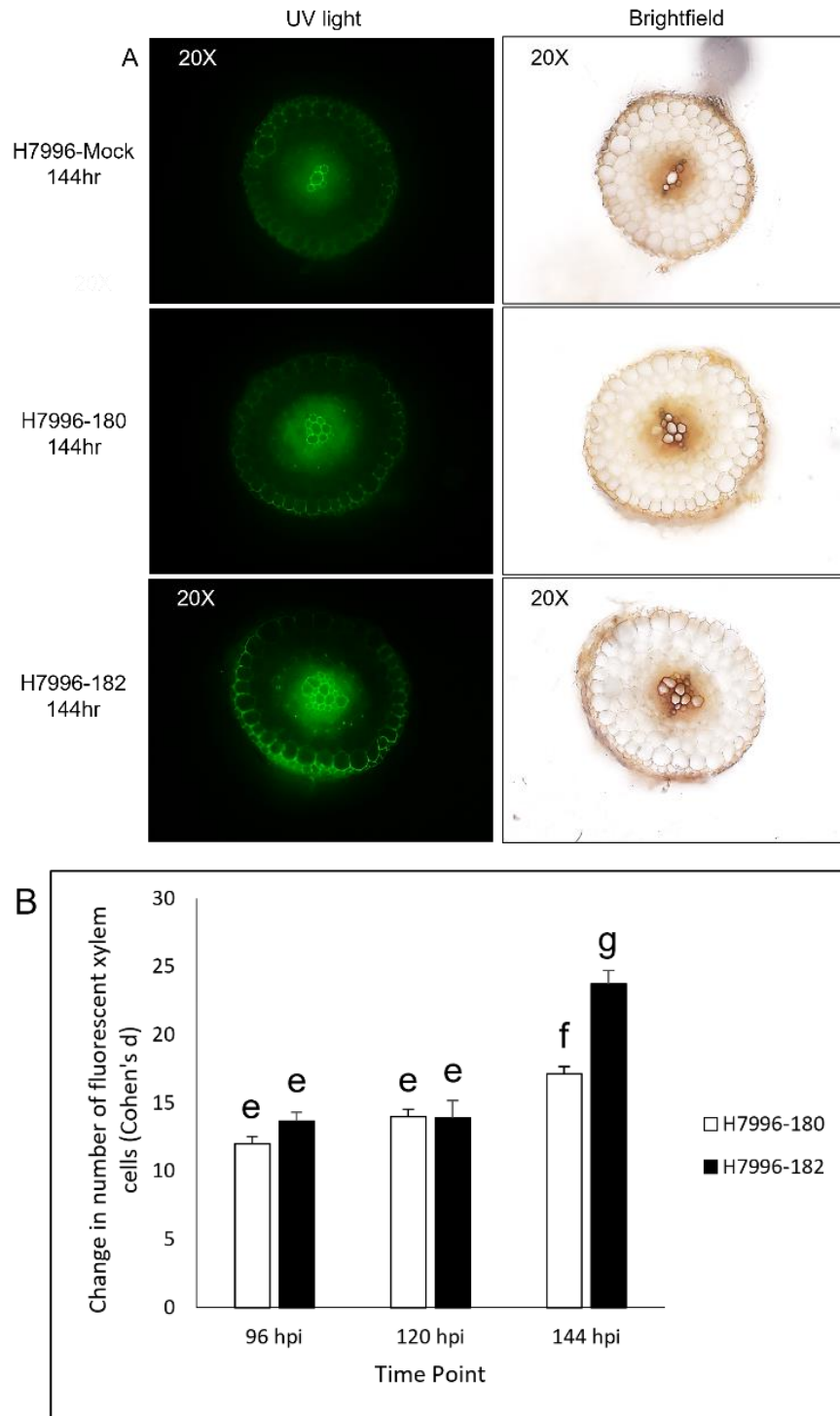


Figure 4.3. Isolates WV180 and WV182 induce cell wall lignification of the xylem vessels in the root of H7996 seedlings. (A) Root cross-section samples stained with Phloroglucinol-HCl at 144 hours after inoculation with either water (mock), isolate WV180, or WV 182 under UV light and brightfield. (B) Barplot describes the quantification of fluorescent xylem cells at 96, 120, and 144 hours after inoculation with WV180 and WV182. Changes in the number of fluorescent cells were calculated as the effect size Cohen's d between the bacterial-treated and mock-treated groups. n=6 plants per isolate; different letters indicate significantly different at $P < 0.05$ from Tukey's posthoc test. H7996, *S. lycopersicum* cv. Hawaii7996. 180, isolate WV180. 182, isolate WV182.

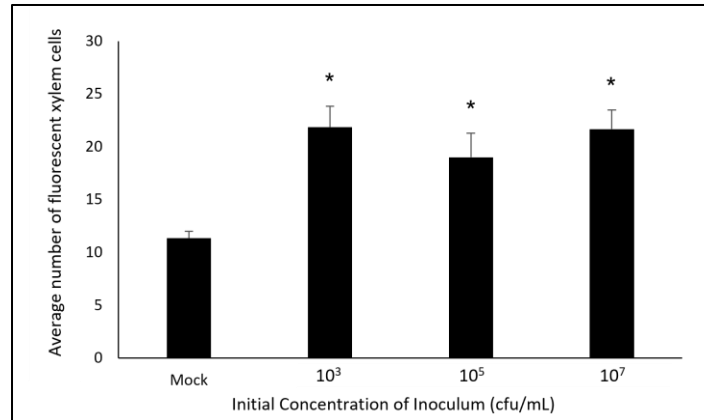


Figure 4.4. The induced number of fluorescent xylem cells is not influenced by the inoculum concentration of WV120. Barplot describes the average number of fluorescent xylem cells in H7996 root at 144 hours after inoculation with either water (mock) or three different concentrations of WV120 inoculum (10^3 , 10^5 , and 10^7 cfu/mL). $n=6$ plants per isolate; different letters indicate significantly different at $P<0.05$ from Tukey's post-hoc test. H7996, *S. lycopersicum* cv. Hawaii7996.

4.4 Conclusions and Future Direction

Our study suggests structural changes in roots in a cell-type-specific manner in response to beneficial bacteria. The two isolates WV180 and WV182 promote root elongation and induce cell wall lignification of the xylem vessels in inoculated roots. Changes in root phenotype do not appear to be influenced by the root compartment where the bacteria colonize and the bacterial concentration during early interaction. Future studies are needed to uncover a possible correlation between the lignification of the xylem vessels and the plant growth-promoting effect induced by the two isolates. Knowledge of cell-type specific responses in plant-microbe interaction is critical for developing crop breeding strategies with enhanced associations with beneficial bacteria while mitigating the detrimental effects of pathogen infection. Below are possible directions for the future works of this study.

Since our results suggest that isolate WV182 stimulates lignification on the root xylem vessels of H7996 seedlings, we hypothesize that the induced lignification enhances host fitness by supporting plant growth or enhancing host resilience against pathogen invasion. Cell wall lignification functions as a critical factor for plant development, providing structural strength for increased physical support and transportation of water (Vanholme et al. 2010; Barros et al. 2015). Lignin is a polymer constituted by monolignols, which chemical structures give rise to three major types of lignin units p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Boerjan, Ralph, and Baucher 2003; Ralph et al. 2004). The composition of monolignols and lignin contents are

determined by plant species, specific cell types, developmental stages, and recently discovered as a defense mechanism of the plant (Voxeur et al. 2015; Sattler and Funnell-Harris 2013; Malinovsky, Fangel, and Willats 2014; Cesarino 2019). We first ask how much and what type of lignin was induced by WV182 at 144hpi. This question will be answered in collaboration with Dr. Clint Chapple's laboratory in the Department of Biochemistry, Purdue University. Briefly, bacterial inoculated roots and mock samples of H7996 seedlings will be sent to The Chapple Lab for subsequent analysis. Quantification of lignin will be performed with Acetyl Bromide and DFRC methods. Identification of lignin contents will be performed with LC-MS. The type of lignin unit and its level of accumulation would hint at potential mechanisms of the host during early interaction with beneficial bacteria.

Our results indicate only specific cell types, the xylem vessels, of inoculated roots were lignified during interaction with isolate WV182. It remains to be investigated whether the endophytic bacteria need to colonize specific cells or tissues to induce the observed host response. To answer this question, we will perform the immunolocalization experiment to visualize bacterial colonization on the root cross-section of H7996 seedlings. Through collaboration with Dr. Jonathan Jacobs from The Ohio State University, we labeled isolate WV182 with GFP utilizing the Tn7-based cloning system. The GFP-labeled strain was confirmed to have similar physiology as the wild-type strain. After inoculation with isolate WV182-GFP, roots will be harvested and fixed in paraffin. Cross-section samples of the prepared root will be obtained and dyed with appropriate antibodies. Interaction with the antibodies leads to the fluorescence of GFP protein on the surface of dead bacteria and can easily be observed under UV illumination.

Kashyap et al. 2022 suggested vasculature lignification functions as an inducible physical barrier in the presence of pathogens, isolating the foreign invaders from further spreading to adjacent cells. To test whether vasculature lignification induced by WV182 contributes to host resistance against pathogens, we will perform the challenge experiment with the vascular pathogen *Ralstonia solanacearum*, a causal agent of bacterial wilt disease in tomato. This pathogen invades plant roots through natural wounds, colonizes the xylem cells, and proliferates inside the vasculature of the infected host (Caldwell et al. 2017). We first need to identify a susceptible tomato variety with a similar cell-type-specific response as H7996 during interaction with isolate WV182. The domesticated tomato H7996 studied in the early results is highly resistant to *R. solanacearum* (Caldwell et al. 2017; French et al. 2018). WV is susceptible to *R. solanacearum*,

but does not have a robust response during interaction with WV182. In the pathogen-challenge experiment, the susceptible tomato variety will be inoculated with the beneficial endophyte WV182, and subsequently with the pathogen *R. solanacearum* at 144hpi. Host resilience will be measured by wilting score assessment at multiple time points. Evidence of the induced lignification enhances host tolerance against *R. solanacearum*, including delayed or reduced wilting symptoms by day ten after being challenged with the pathogen.

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APPENDIX A. CHAPTER 2 SUPPLEMENTAL FIGURES AND TABLES

FIGURES

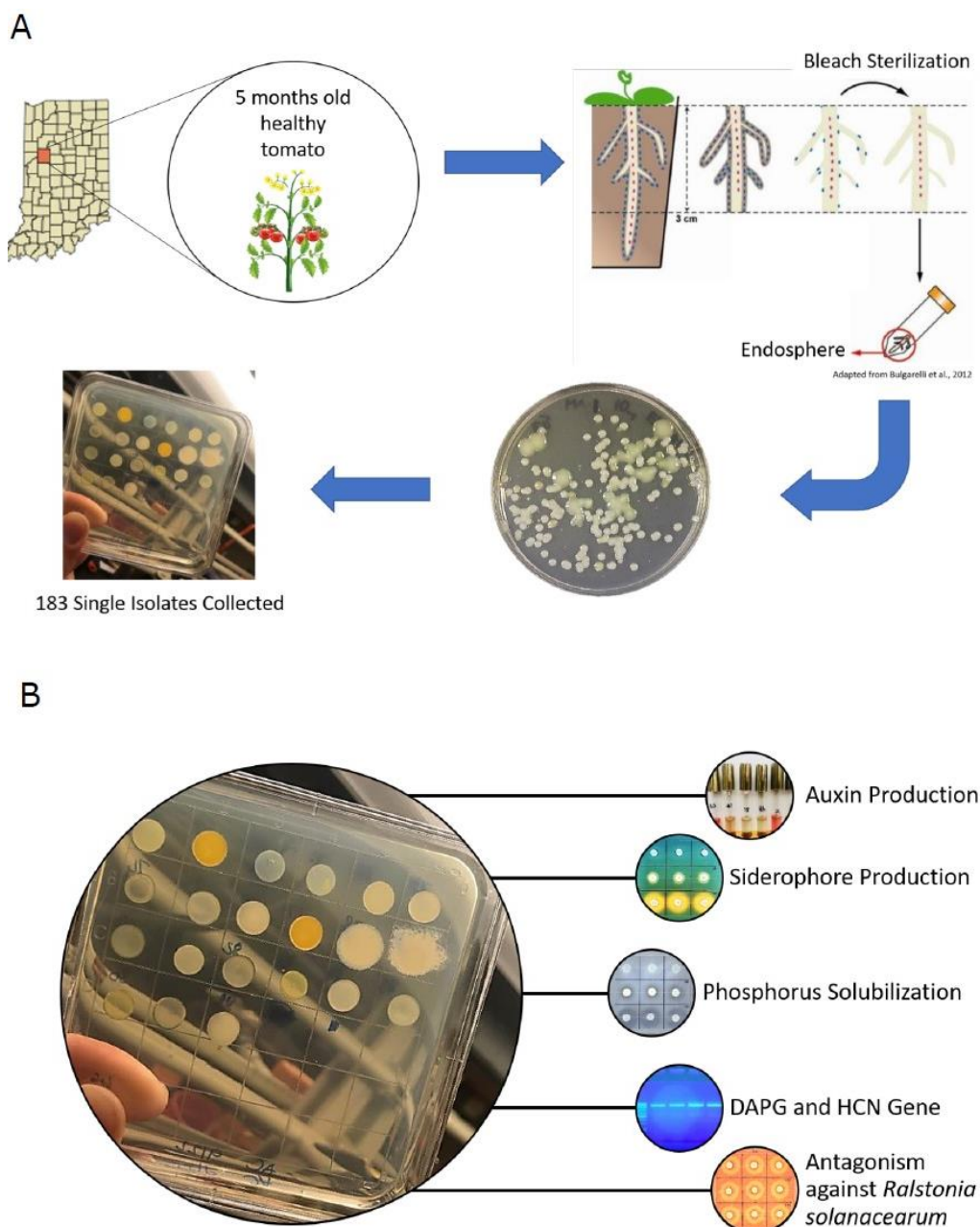


Figure A2.1. Schematic presentation for methods of endophyte isolation, building collection, and screening for multiple *in vitro* functions (see MaterialsMethods for details). A. Healthy five-month-old field-grown roots of two tomato genotypes H7996 and WV were harvested in central Indiana. Root tissues were surface sterilized and homogenized with sterile 1x PBS. Homogenized liquid was dilution plated on different selection media. Part of figure S1A was adapted from Bulgarelli et al. 2012. B. Bacterial endophytes were subsequently screened for the *in vitro* functional traits shown in the image.

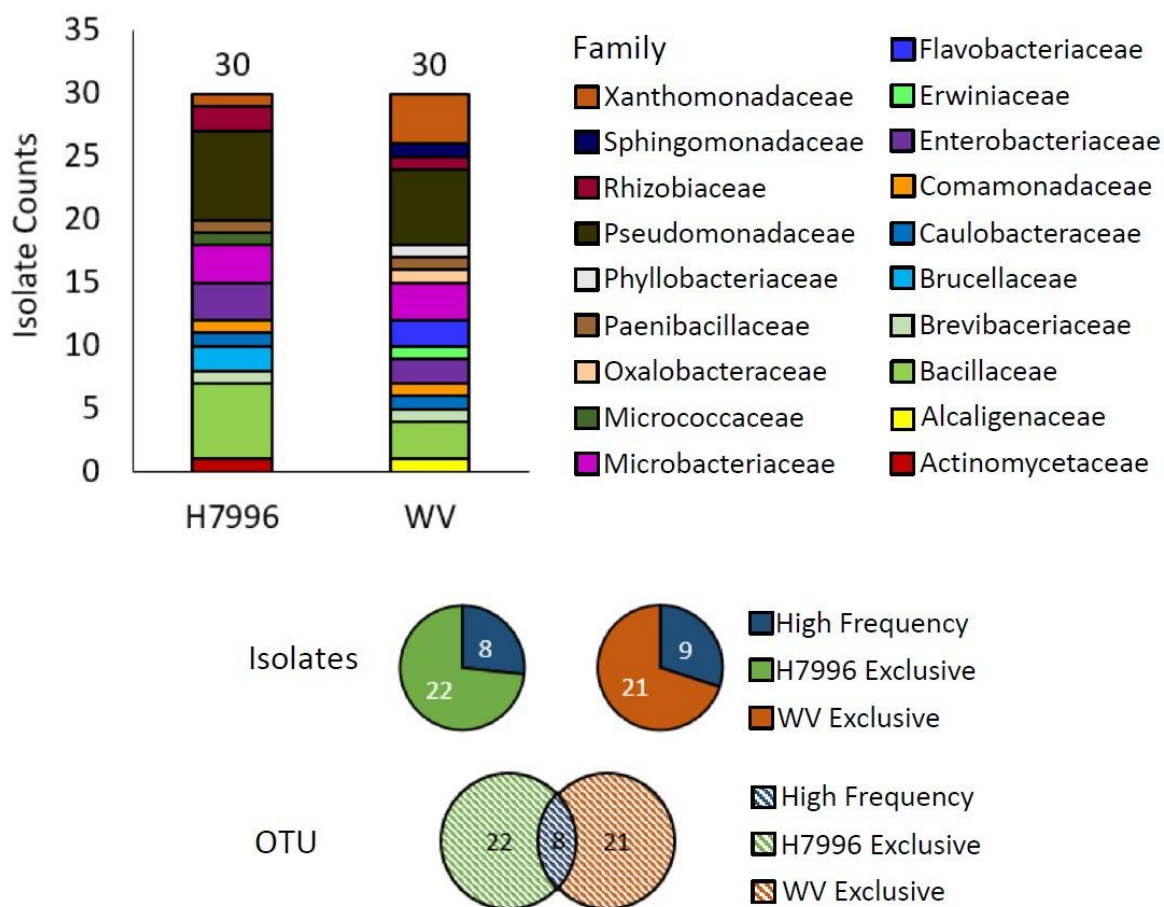


Figure A2.2. Stacked barplot of isolate counts at family level of the 60 isolates selected for *in vitro* functional characterization experiment and their host origins. Pie charts show the host origin for the endophytes as isolates and OTU.

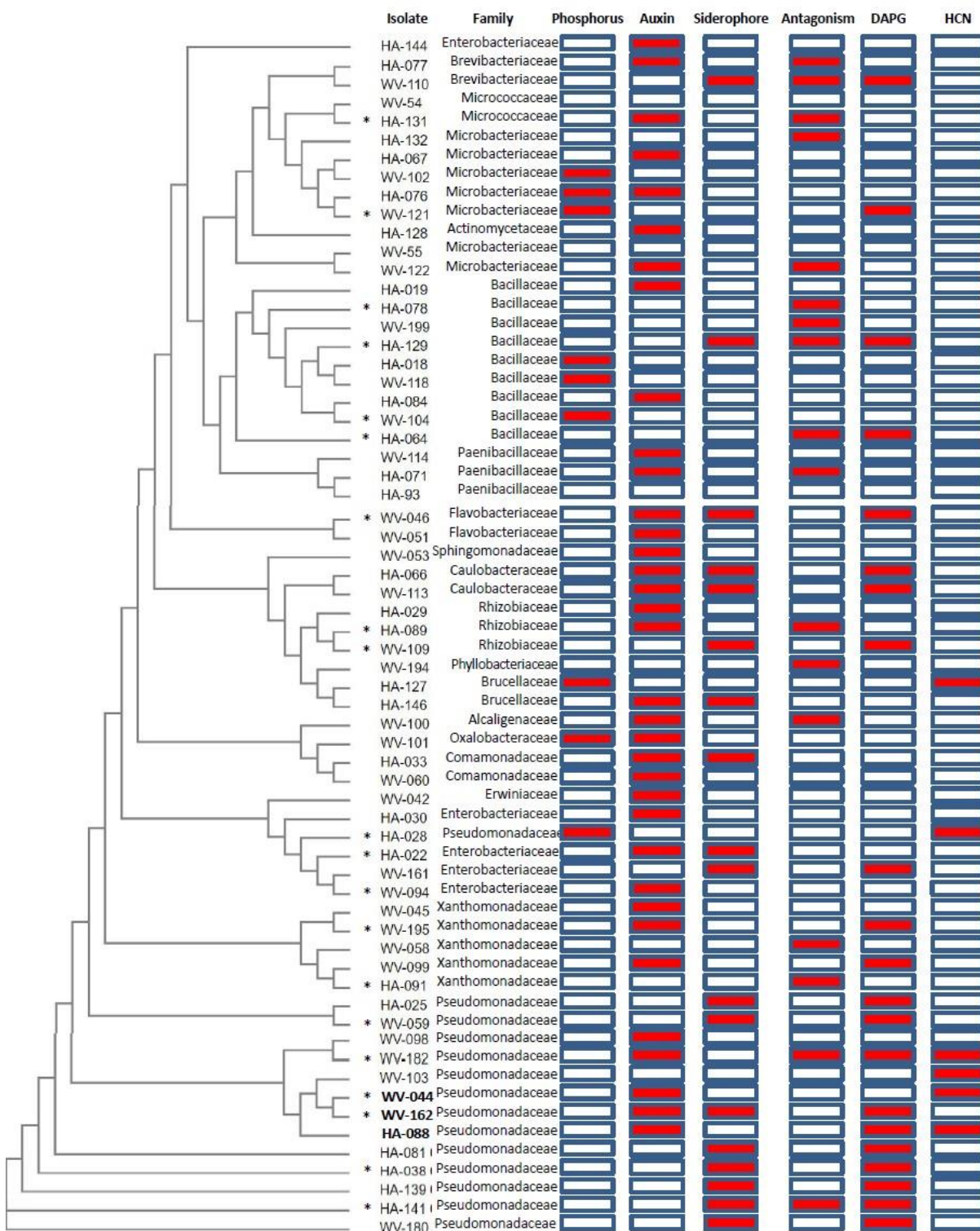


Figure A2.3. Phylogenetic tree of the 60 isolates selected for *in vitro* functional characterization experiment and their functional traits. The isolates are grouped based on their similarity of their 16S rRNA gene sequence. The six columns of boxes showing the functional traits that were tested positive (filled) and negative (empty) of the isolate described on the same row. Functional groups were determined based on the first four traits. The three isolates with the same OTU classification mentioned in the text, WV-044, WV-162, and HA-088, are bolded. Asterisks indicates the 20 isolates chosen for *in planta* binary interaction experiment.

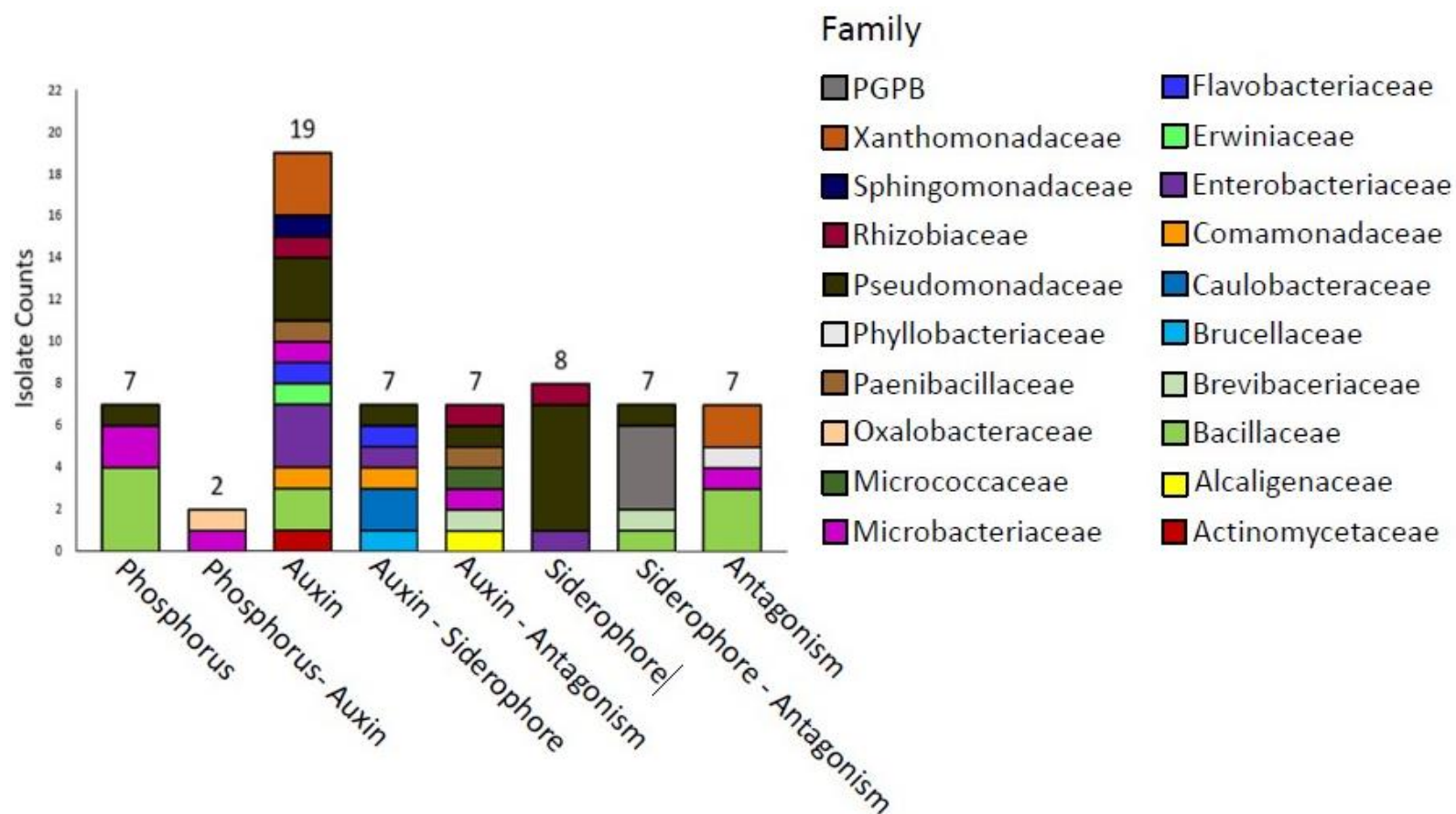
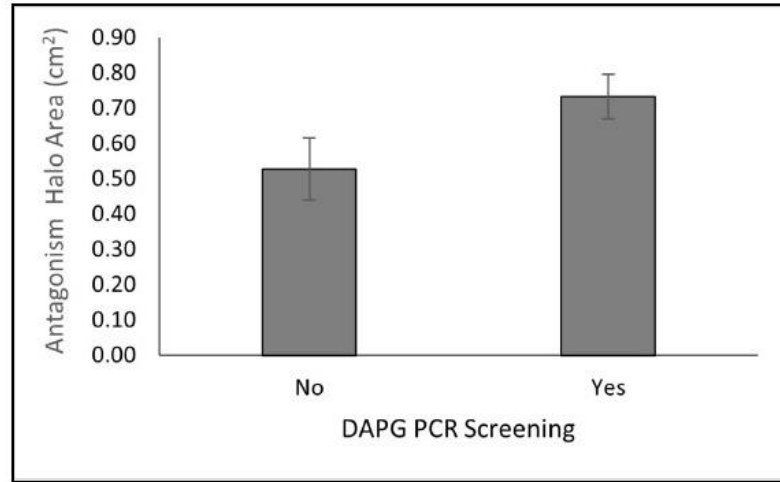


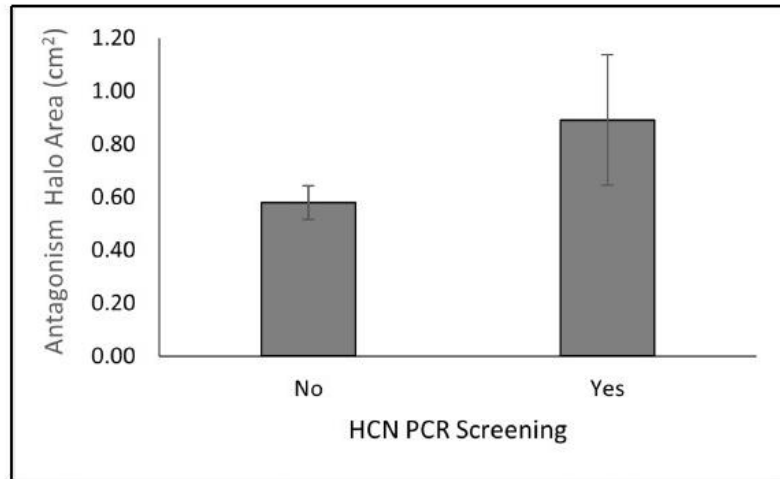
Figure A2.4. Number of isolates at family level in each of the functional groups in Figure 4.

A.



Antagonism-DAPG: $R^2 = 0.0159$, $P > 0.05$ Kruskal-Wallis test

B.



Antagonism-HCN: $R^2 = 0.0107$, $P > 0.05$ Kruskal-Wallis test

Figure A2.5. No correlation was observed between the presence of the *phlD* gene (A; DAPG PCR Screening) or the *hcnAB* gene (B; HCN PCR screening) and the antagonism trait.

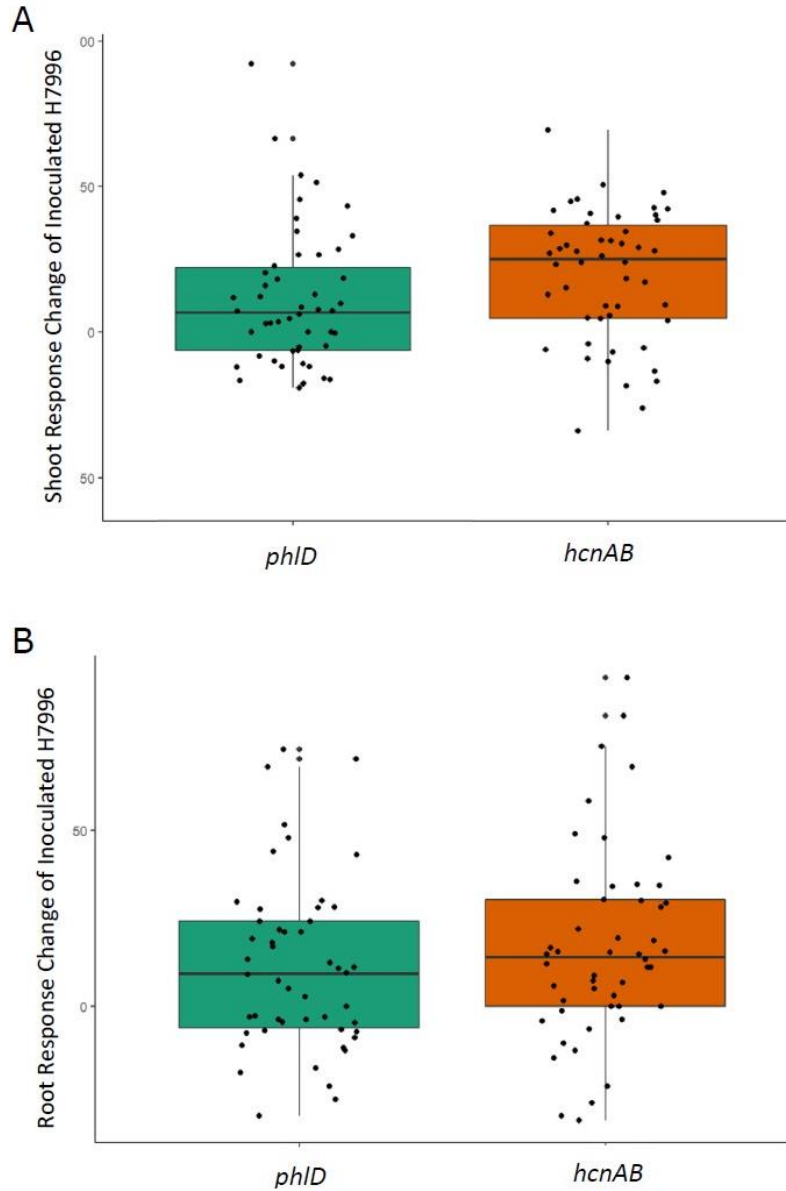


Figure A2.6. Scatterplots showing plant growth promoting effect between two qualitative functional traits (presence of *phlD* and *hcnAB* genes in the genome) of the 20 isolates tested for plant-binary interaction experiment. Each dot represents the response rate of one inoculated plant. Response rate was calculated by the change in shoot (A) or root (B) weight of inoculated H7996 compared with mock-treated plants of the same genotype. $P > 0.05$ using the Mann-Whitney U test.

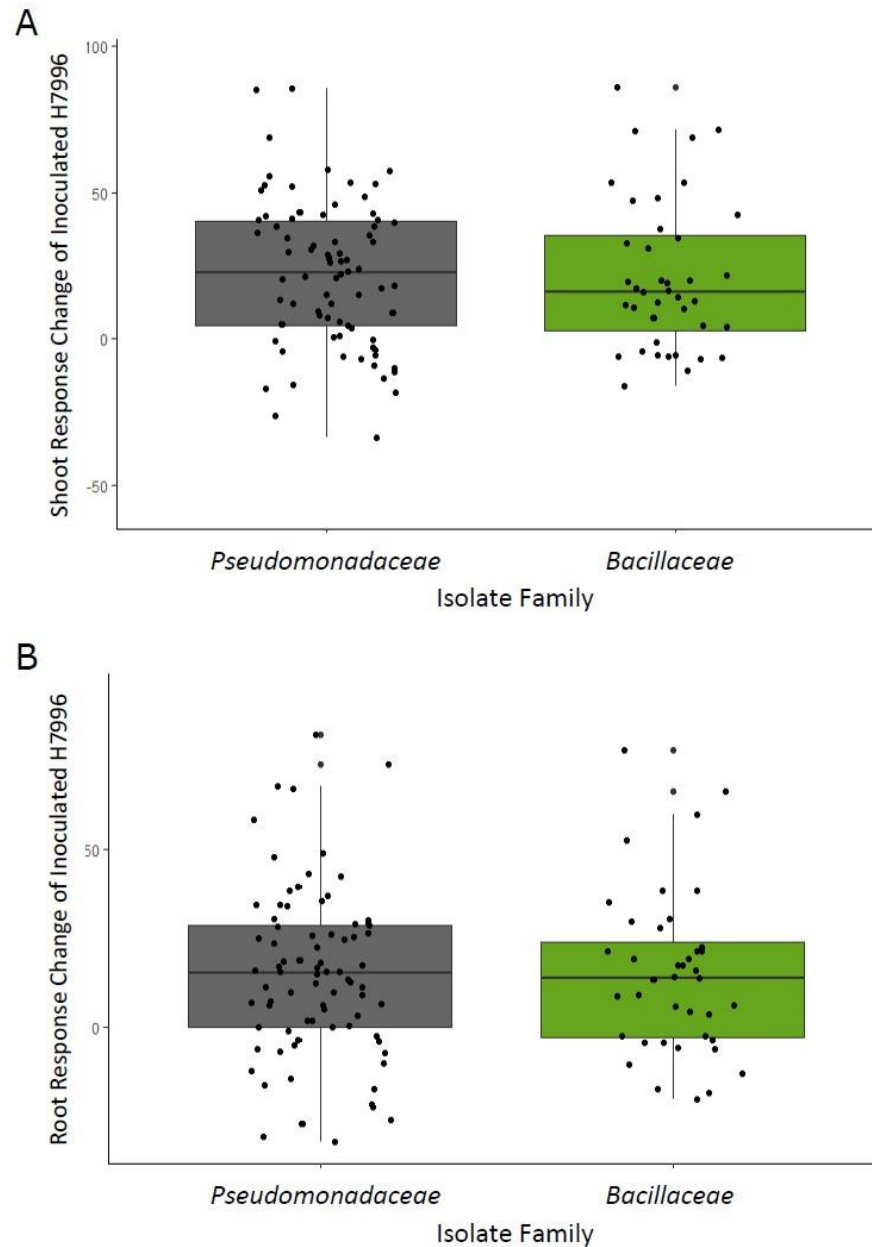


Figure A2.7. Scatterplots of the plant growth promoting effect between members of the two most common bacterial families in our culturable endophyte collection, *Pseudomonadaceae* and *Bacillaceae*. Each dot represents the response rate of one inoculated plant. Response rate was calculated by the change in shoot (A) or root (B) weight of inoculated H7996 compared with mock-treated plants of the same genotype. No statistical difference in responses between the two families was observed. $P > 0.05$ using the Mann-Whitney U test.

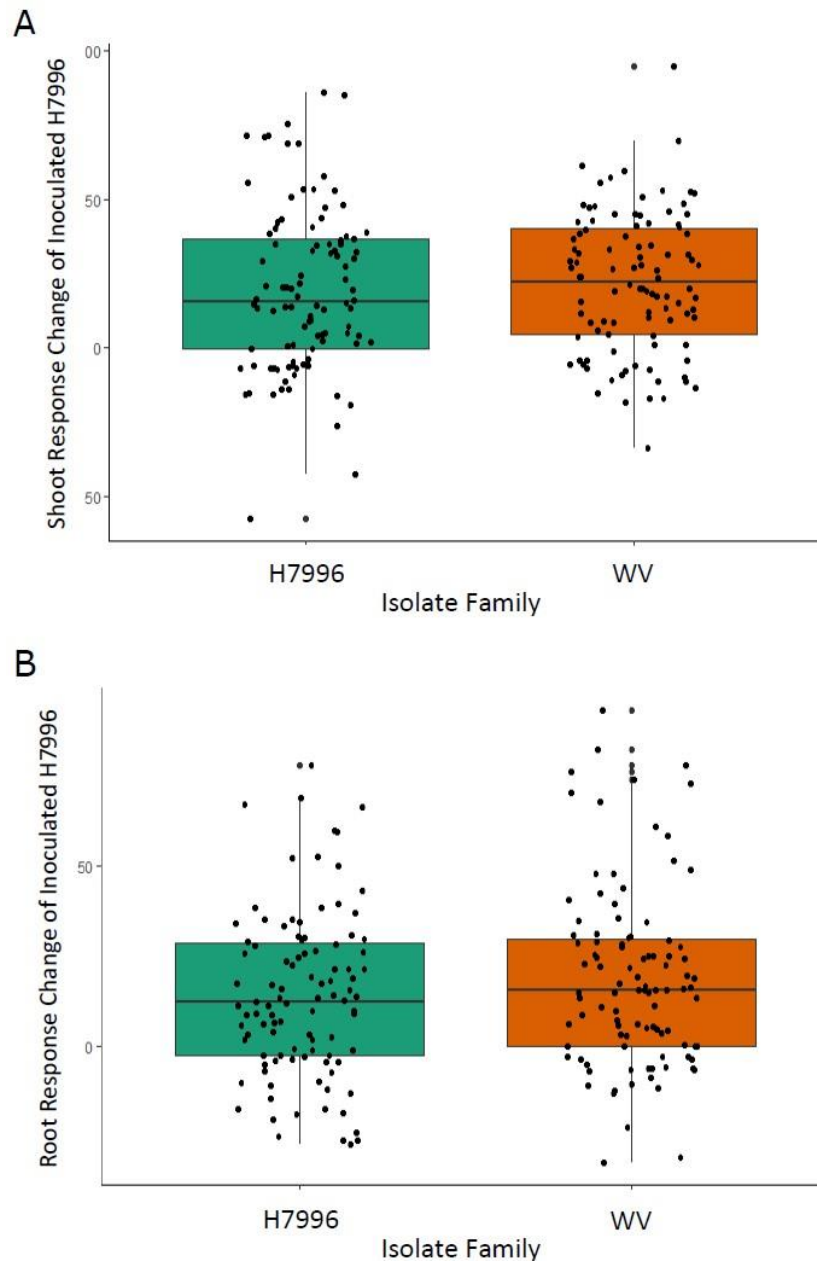


Figure A2.8. Scatterplots showing plant growth promoting effect between the two host origins of the 20 isolates tested for plant-binary interaction experiment. Ten plants were inoculated with each isolate. Each dot represents the response rate of one inoculated plant. Response rate was calculated by the change in A. shoot or B. root weight of inoculated H7996 compared with mock-treated plants of the same genotype. Abbreviations: H7996 – *S. lycopersicum* cv. Hawaii7996. WV – *S. pimpinellifolium* accession WestVirginia700. $P > 0.05$ using the Mann-Whitney U test.

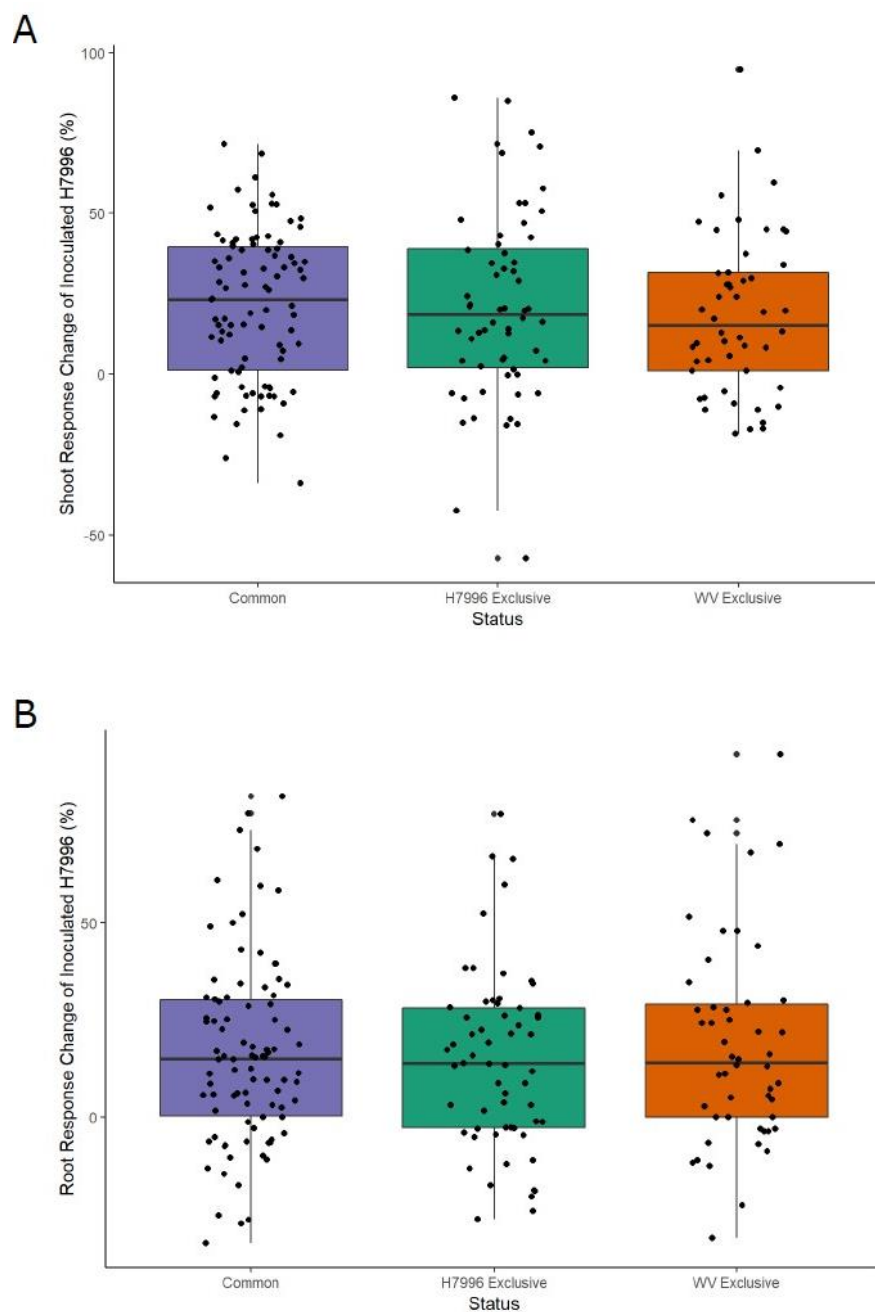


Figure A2.9. Scatterplots showing plant growth promoting effect between the three OTU classifications of the 20 isolates tested for plant-binary interaction experiment. Each dot represents the response rate of one inoculated plant. Response rate was calculated by the change in A. shoot or B. root weight of inoculated H7996 compared with mock treated plants of the same genotype. Abbreviations: H7996 – *S. lycopersicum* cv. Hawaii7996. WV – *S. pimpinellifolium* accession WestVirginia700. $P > 0.05$ using the Kruskal-Wallis test.

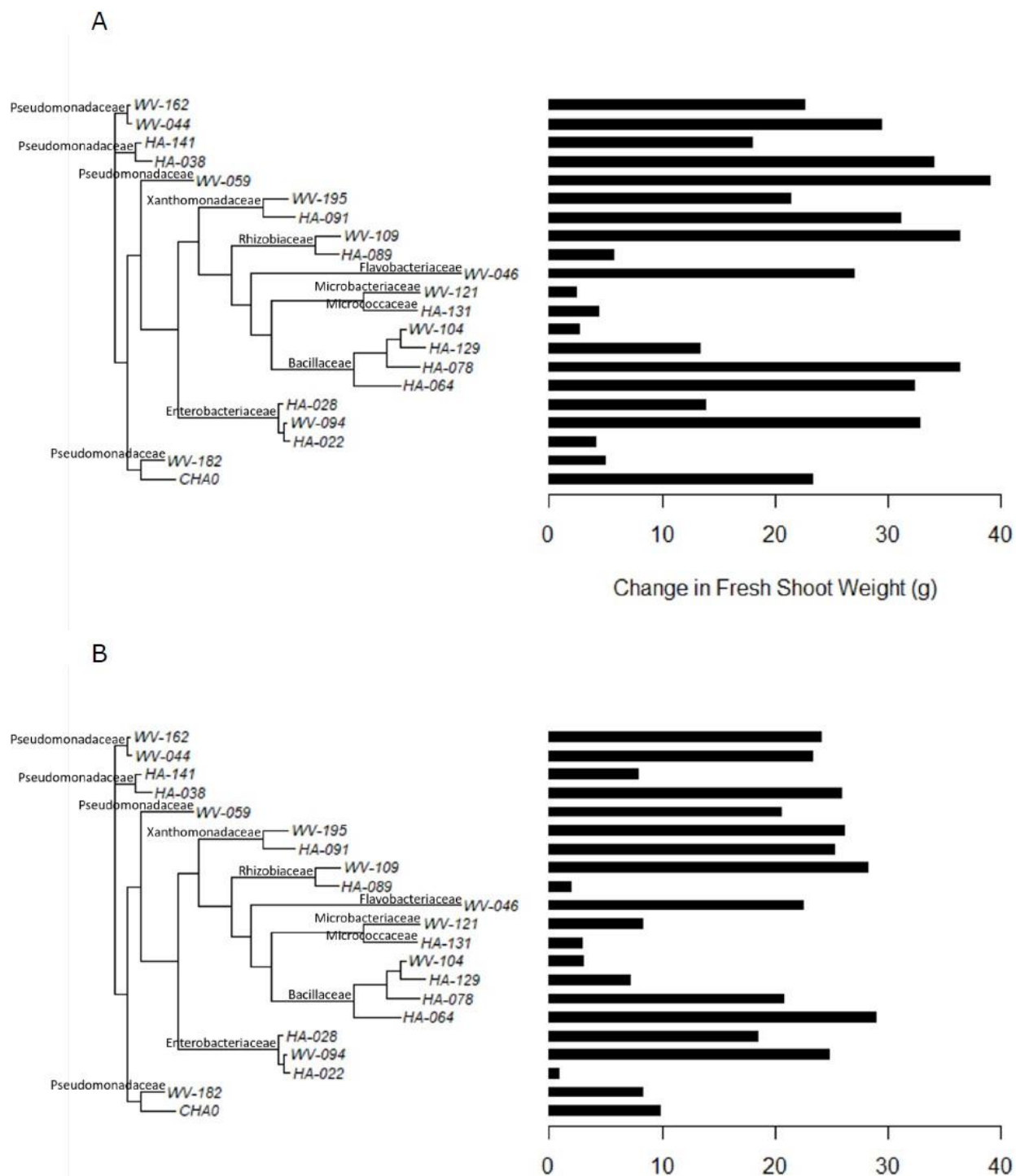


Figure A2.10. Plant response rate of fresh shoot (A) and root (B) weight of the endophytes clustered by isolate phylogeny. Bars represent changes in values of bacteria-inoculated plants when compared with mock-treated plant of the same genotype (n = 10 plants per isolate).

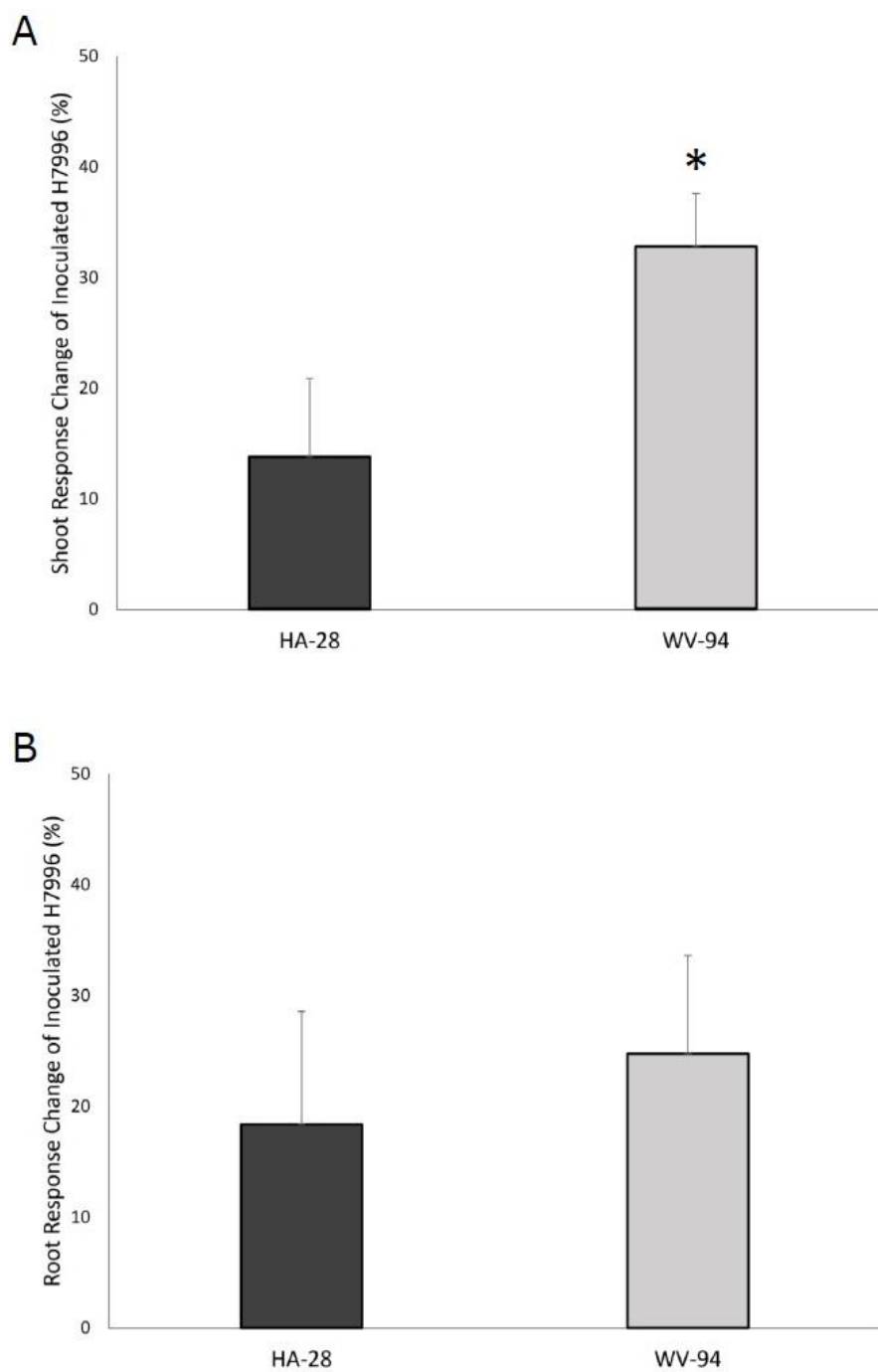
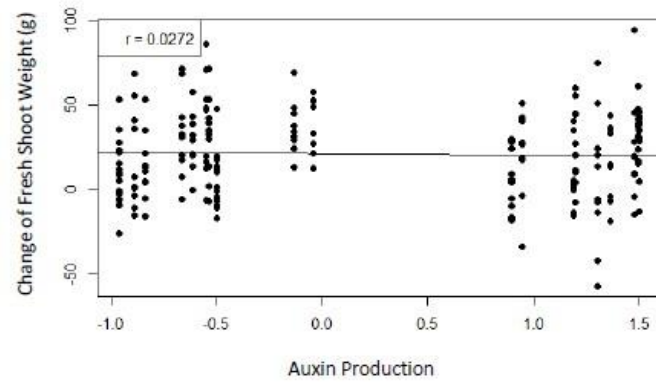


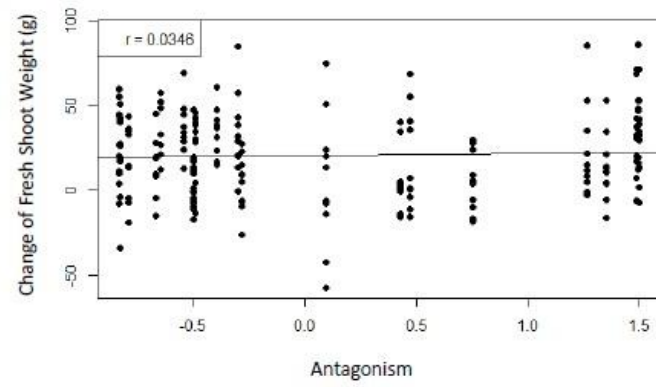
Figure A2.11. Comparison of host response rate between the two isolates with similar OTU classification, HA-28 and WV-94. Barplots showing the change in fresh shoot (A) and root (B) weight of plants inoculated with either HA-28 or WV-98 compared to mock treated plants of the same genotype (n = 10 plants per isolate, asterisk indicates significantly different at $P < 0.05$ from student's t-test).

Figure A2.12. Individual *in vitro* traits do not predict the impact of isolates on shoot growth. Scatterplots showing linear regression between individual bacterial trait (x-axis) of A. Auxin Production, B. Antagonism, C. Phosphorus Solubilization, and D. Siderophore Production, and the impact of the isolate on fresh shoot weight of inoculated plants (y-axis). Each dot represents the response rate of one inoculated plant. Response rate was calculated by the change in shoot weight of inoculated H7996 compared with mock-treated plants of the same genotype.

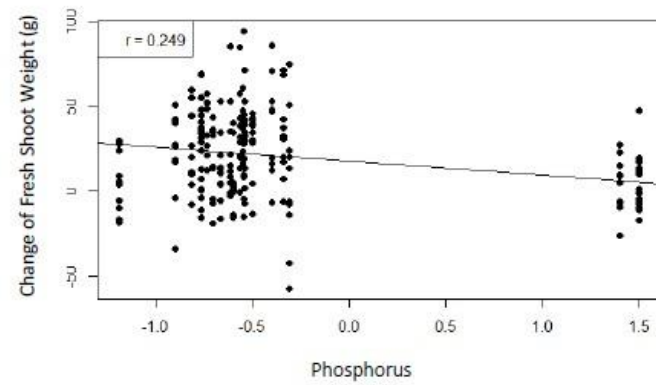
A



B



C



D

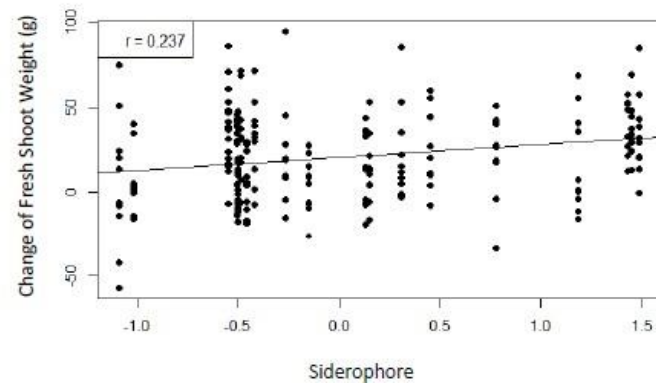
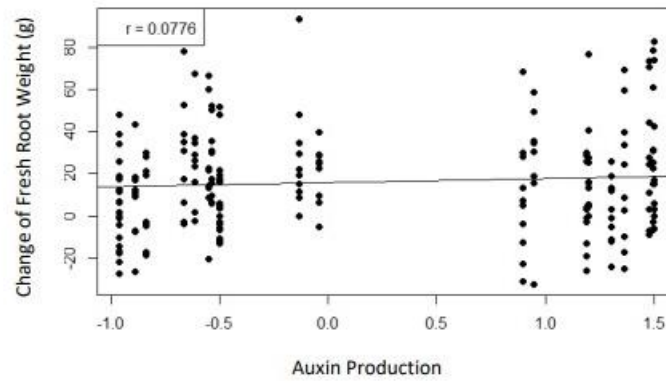
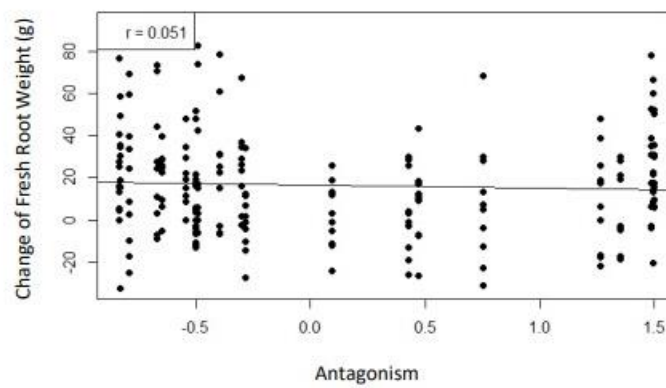


Figure A2.13. Individual *in vitro* traits do not predict the impact of isolates on root growth. Scatterplots showing linear regression between individual bacterial trait (x-axis) of A. Auxin Production, B. Antagonism, C. Phosphorus Solubilization, and D. Siderophore Production, and the isolates' impact on fresh root weight of inoculated plants (y-axis). Each dot represents the response rate of one inoculated plant. Response rate was calculated by the change in shoot weight of inoculated H7996 compared with mock-treated plants of the same genotype.

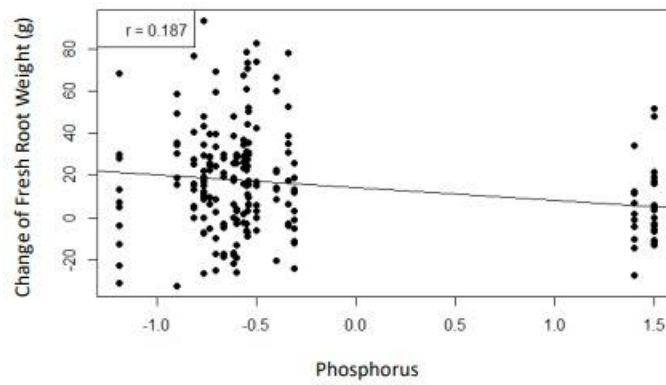
A



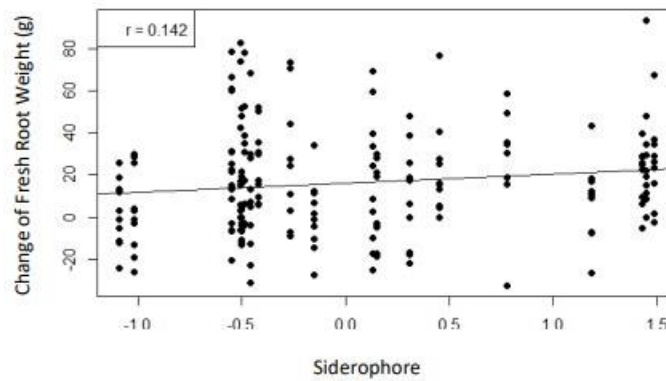
B



C



D



TABLES

***Table A2.1.** Summary of sequencing result, taxonomic identification, and OTU classification of 183 bacterial endophytes in the collection. Isolates in bold were used for the functional characterization experiment.

***Table A2.2.** Summary of functional group categorization of the 64 tested isolates (60 endophytes + four control bacteria).

***Table A2.3.** *In planta* growth promotion results.

***Tables A2.1-3 are too large to be included in this document. They are available online at <https://academic-oup-com.ezproxy.lib.purdue.edu/jxb/article/73/16/5758/6590292> and are labeled Supplementary Tables 1, 2, and 3, respectively.**

Table A2.4. Phylogenetic signal metrics for bacterial effects on plant growth.

	K	P_(K)	λ	P_(λ)
Fresh Shoot Weight	0.0862	0.781	6.61E-05	1.000
Fresh Root Weight	0.218	0.108	0.341	0.240

Table A2.5. Results of regression analysis of plant response to the 21 selected isolates in plant-binary interaction experiment. We summarize the following outputs for each model Akaike Information Criteria corrected for small sample size (AICc), R², and P.

Model 1	Fresh Shoot Weight = Auxin Production
Model 2	Fresh Shoot Weight = Group
Model 3	Fresh Shoot Weight = Auxin Production + Group
Model 4	Fresh Shoot Weight = Auxin Production * Group
Model 5	Fresh Shoot Weight = Auxin Production + Group + Auxin Production * Group
Model 6	Fresh Shoot Weight = Phosphorus + Auxin Production + Siderophore + Antagonism
Model 7	Fresh Shoot Weight = Phosphorus + Auxin Production + Siderophore + Antagonism + Group
Model 8	Fresh Shoot Weight = Phosphorus + Auxin Production + Siderophore + Group
Model 9	Fresh Shoot Weight = Phosphorus + Group
Model 10	Fresh Root Weight = Auxin Production
Model 11	Fresh Root Weight = Group
Model 12	Fresh Root Weight = Auxin Production + Group
Model 13	Fresh Root Weight = Auxin Production * Group
Model 14	Fresh Root Weight = Auxin Production + Group + Auxin Production * Group
Model 15	Fresh Root Weight = Phosphorus + Auxin Production + Siderophore + Antagonism
Model 16	Fresh Root Weight = Phosphorus + Auxin Production + Siderophore + Antagonism + Group
Model 17	Fresh Root Weight = Phosphorus + Auxin Production + Siderophore + Group
Model 18	Fresh Root Weight = Phosphorus + Group

Response	Models	AICc	R ²	P
Fresh Shoot Weight	1	1960.82	-0.00407	0.6952
	2	1917.83	0.2029	1.52E-09
	3	1919.98	0.1991	4.98E-09
	4	1930.66	0.1859	5.28E-07
	5	1930.66	0.1859	5.28E-07
	6	1944.63	0.07987	0.000156
	7	1924.41	0.1911	4.34E-08
	8	1924.41	0.1911	4.34E-08
	9	1920	0.199	5.02E-09
Fresh Root Weight	10	1960.82	0.001241	0.263
	11	1917.83	0.124	1.00E-05
	12	1919.98	0.1207	2.35E-05
	13	1930.66	0.1001	0.001132
	14	1930.66	0.1001	0.001132
	15	1944.63	0.03188	0.02152
	16	1924.41	0.1128	0.000119
	17	1924.41	0.1128	0.000119
	18	1920	0.1211	2.26E-05

APPENDIX B. CHAPTER 3 SUPPLEMENTAL FIGURES AND TABLES

FIGURES

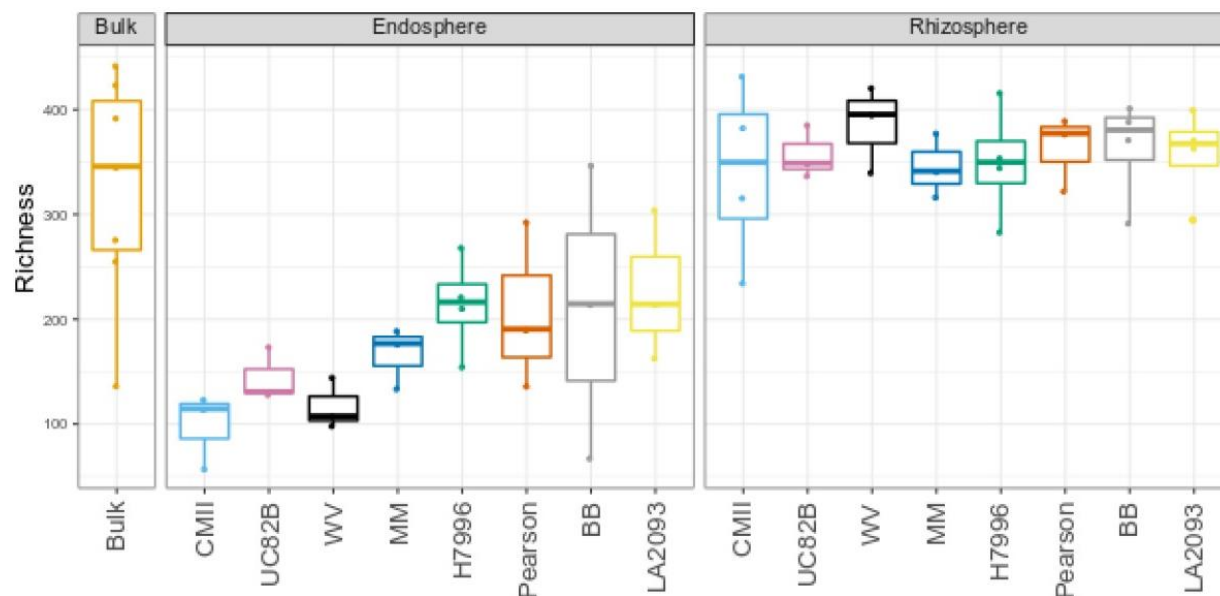


Figure B3.1. Bacterial community richness varies across root compartments. Boxplot of richness of rhizospheres and endospheres of all eight genotypes and bulk (unplanted) soil. Abbreviations: CMII – *S. lycopersicum* cv. Castlemart II. WV – *S. pimpinellifolium* acc. WestVirginia700. UC82B – *S. lycopersicum* cv. UC82B. MM – *S. lycopersicum* cv. Money Maker. BB – *S. lycopersicum* cv. Bonnie Best. Pearson – *S. lycopersicum* cv. Pearson. LA2093 – *S. pimpinellifolium* acc. LA2093. H7996 – *S. lycopersicum* cv. Hawaii7996.

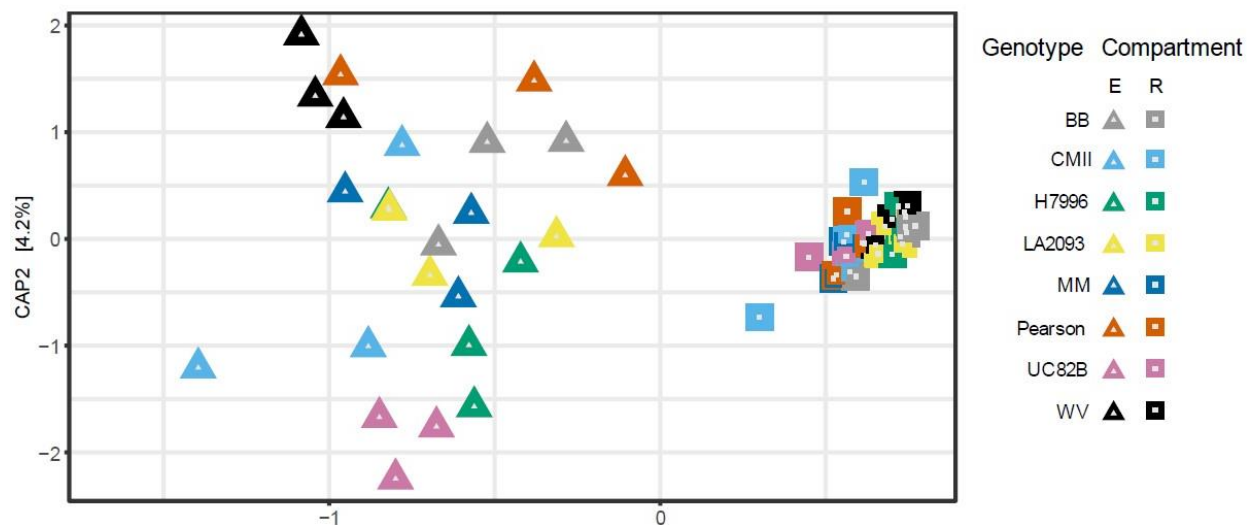


Figure B3.2. Canonical Analysis of Principle Coordinates (CAP) of all eight genotypes with compartment and genotype as constraining factors. Abbreviations: E – endosphere. R – rhizosphere. CMII – *S. lycopersicum* cv. Castlemart II. WV – *S. pimpinellifolium* acc. WestVirginia700. UC82B – *S. lycopersicum* cv. UC82B. MM – *S. lycopersicum* cv. Money Maker. BB – *S. lycopersicum* cv. Bonnie Best. Pearson – *S. lycopersicum* cv. Pearson. LA2093 – *S. pimpinellifolium* acc. LA2093. H7996 – *S. lycopersicum* cv. Hawaii7996.

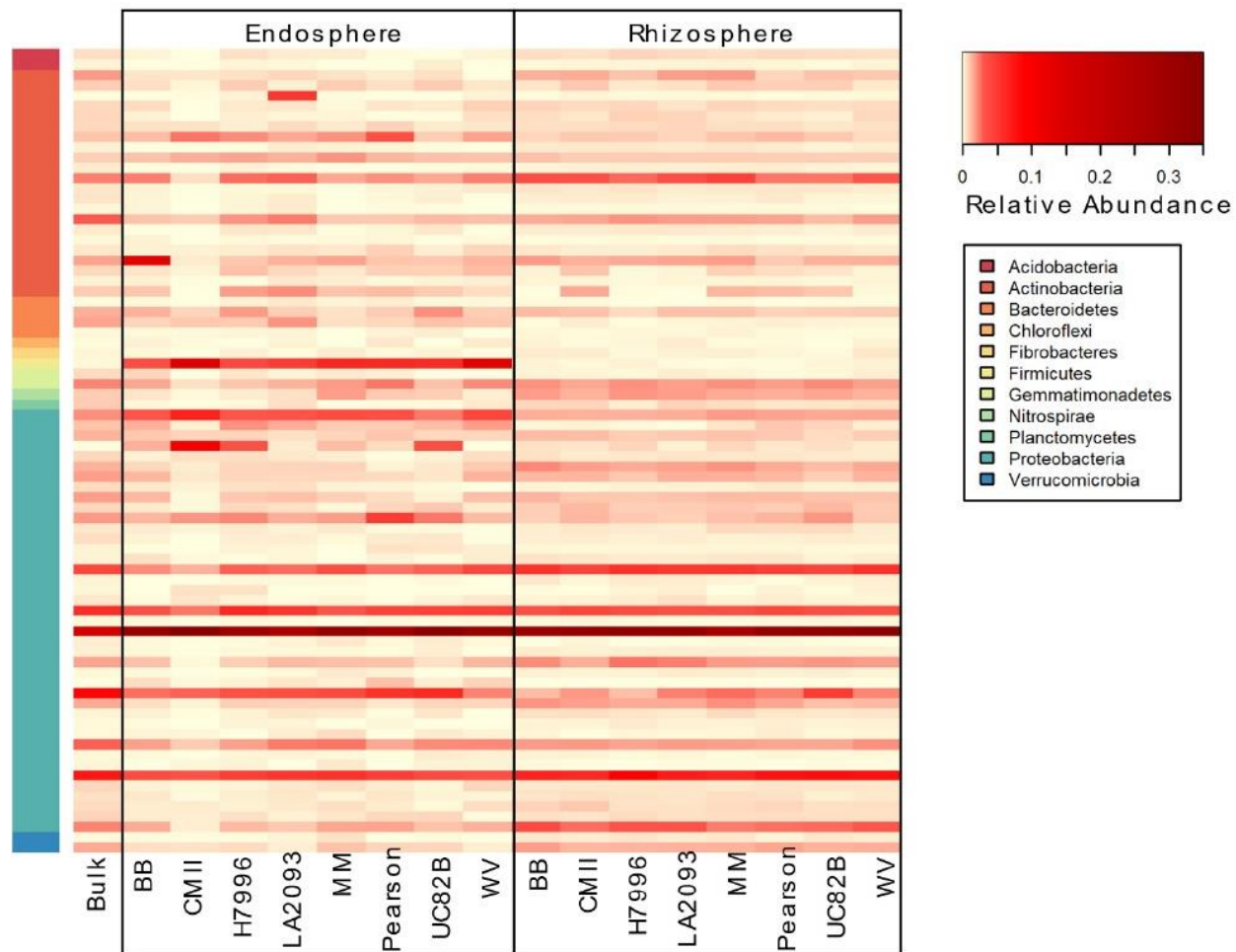


Figure B3.3. Heatmap showing average relative abundance of bacterial families in the bulk soil and across the root endosphere and rhizosphere of all eight genotypes. Sidebar colors represent phyla of each family. Heatmap shows families that make up over 0.1% average relative abundance (78 out of 122). CMII – *S. lycopersicum* cv. Castlemart II. WV – *S. pimpinellifolium* acc. WestVirginia700. UC82B – *S. lycopersicum* cv. UC82B. MM – *S. lycopersicum* cv. Money Maker. BB – *S. lycopersicum* cv. Bonnie Best. Pearson – *S. lycopersicum* cv. Pearson. LA2093 – *S. pimpinellifolium* acc. LA2093. H7996 – *S. lycopersicum* cv. Hawaii7996.

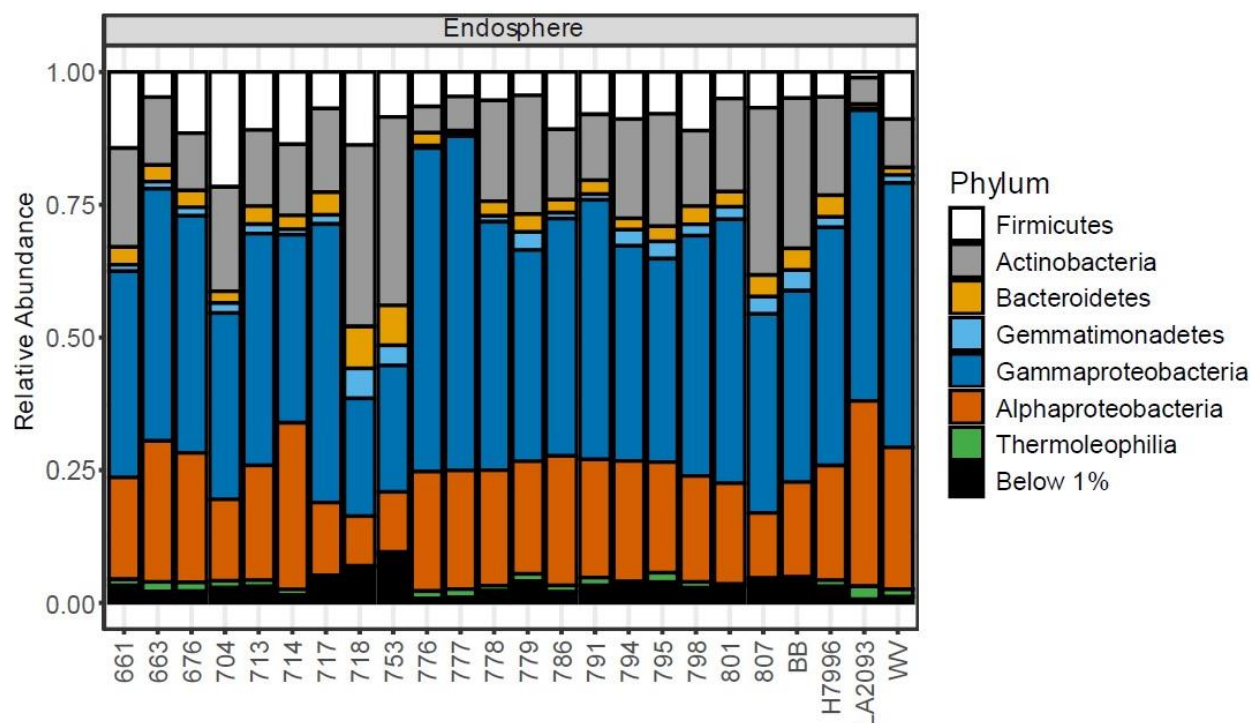


Figure B3.4. Stacked barplot of relative abundance of bacterial taxa at phylum level for RILs, parental lines, and an additional *S. lycopersicum* (BB) and *S. pimpinellifolium* (LA2093) genotype. Phyla with less than 1% average abundance across genotypes were grouped together and represented as “Below 1%”. Numbered genotypes indicate RILs. BB and LA2093 included to show variation due to species. Abbreviations: WV – *S. pimpinellifolium* acc. WestVirginia700. LA2093 – *S. pimpinellifolium* acc. LA2093. H7996 – *S. lycopersicum* cv. Hawaii7996.

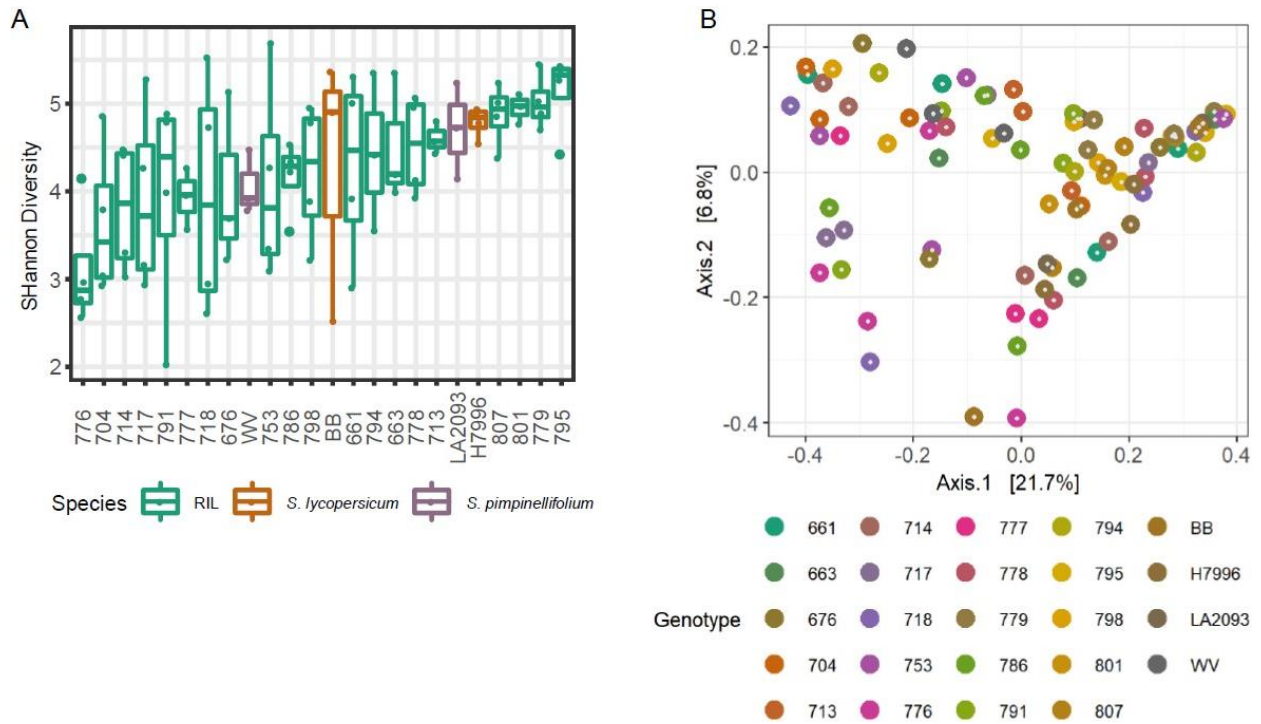


Figure B3.5. Variation in endosphere diversity across twenty recombinant inbred lines (RILs) derived from H7996 and WV. A) Boxplot of Shannon diversity and B) Principle coordinate analysis of Bray-Curtis distance across RILs, parental lines, and an additional *S. lycopersicum* (BB) and *S. pimpinellifolium* (LA2093) genotype. Numbered genotypes indicate RILs. BB and LA2093 included to show variation due to species. Abbreviations: WV – *S. pimpinellifolium* acc. WestVirginia700. LA2093 – *S. pimpinellifolium* acc. LA2093. H7996 – *S. lycopersicum* cv. Hawaii7996.

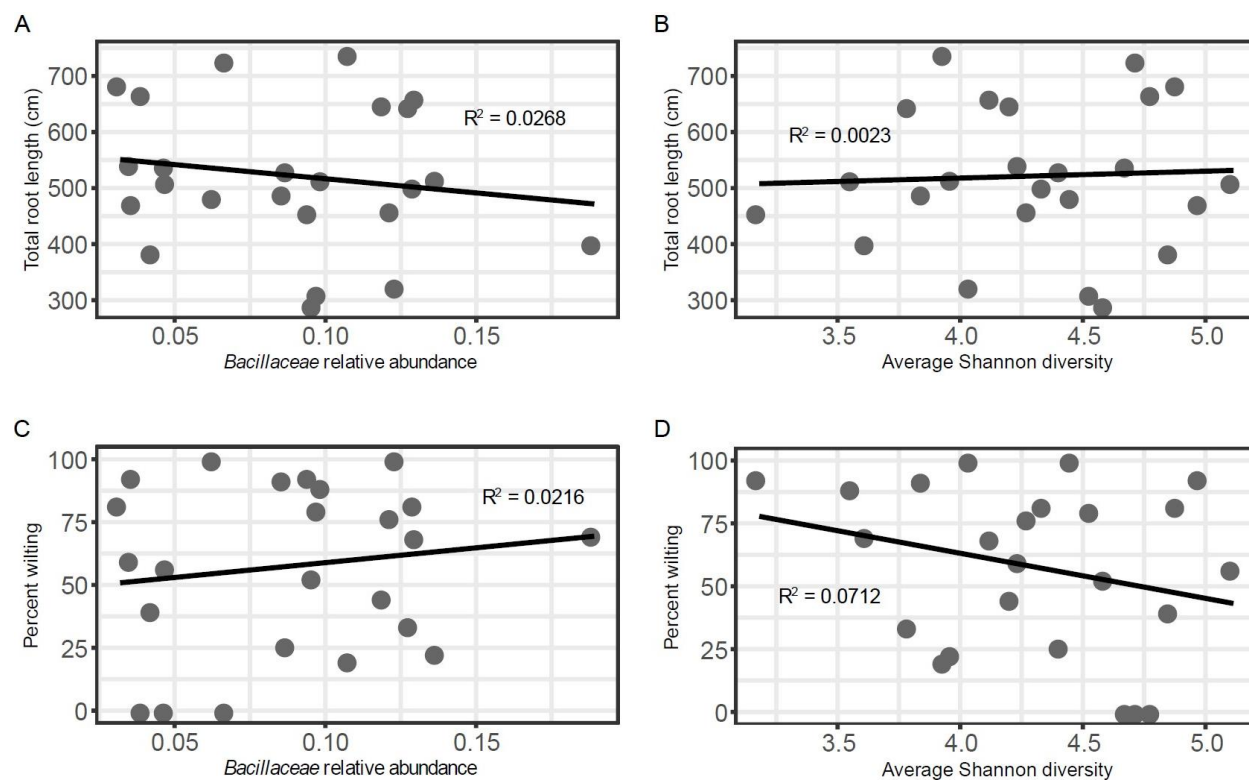


Figure B3.6. Tomato root architecture and resistance to *Ralstonia solanacearum* do not correlate with bacterial microbiome traits. Linear correlation between average total root length and (A) relative abundance of *Bacillaceae* and (B) Average Shannon diversity in the root endosphere for each measured RIL and parental line as well as BB and LA2093 genotypes. Linear correlation between percent wilting in response to *Ralstonia solanacearum* infection and (C) relative abundance of *Bacillaceae* and (D) Average Shannon diversity for each measured RIL and parental line as well as BB and LA2093 genotypes. R^2 values are shown for each panel.

TABLES

Table B3.1. Soil characteristics.

<i>Soil</i>	<i>% OM</i>	<i>Bray-1 P</i> (<i>ppm</i>)	<i>K</i> (<i>ppm</i>)	<i>Mg</i> (<i>ppm</i>)	<i>Ca</i> (<i>ppm</i>)	<i>pH</i>	<i>CEC</i> (<i>meq/100g</i>)	<i>NO³-N</i> (<i>ppm</i>)
<i>Potting Mix/Field soil</i>	6.8 (±0.4)	40.3 (±0.3)	137 (±4.5)	285 (±2.9)	1483 (±33.3)	6.8 (±0.03)	10.7 (±0.3)	26 (±0.6)
<i>Field soil</i>	2.6 (±0.1)	61.7 (±1.2)	173 (±3.8)	243 (±3.3)	1567 (±33.3)	7.4 (±0.03)	10.3 (±0.2)	7 (±0.0)

Table B3.2. *S. lycopersicum*, *S. pimpinellifolium* lines and RILs.

Genotype	Species/RIL	Rhizo?	Endo?	Growth Promotion Assay?	Percent wilting
H7996	<i>Solanum lycopersicum</i>	✓	✓	yes	0
WV	<i>S. pimpinellifolium</i>	✓	✓	yes	100
LA2093	<i>S. pimpinellifolium</i>	✓	✓	no	0
BB	<i>S. lycopersicum</i>	✓	✓	no	100
UC82B	<i>S. lycopersicum</i>	✓	✓	no	na
Pearson	<i>S. lycopersicum</i>	✓	✓	no	na
Money Maker (MM)	<i>S. lycopersicum</i>	✓	✓	no	na
Castlemart II (CMII)	<i>S. lycopersicum</i>	✓	✓	no	na
661	H7996xWV RIL		✓	yes	77
663	H7996xWV RIL		✓	yes	0
676	H7996xWV RIL		✓	yes	23
704	H7996xWV RIL		✓	yes	70
713	H7996xWV RIL		✓	no	53
714	H7996xWV RIL		✓	yes	34
717	H7996xWV RIL		✓	yes	45
718	H7996xWV RIL		✓	yes	20
753	H7996xWV RIL		✓	yes	89
776	H7996xWV RIL		✓	yes	93
777	H7996xWV RIL		✓	yes	92
778	H7996xWV RIL		✓	yes	100
779	H7996xWV RIL		✓	no	93
786	H7996xWV RIL		✓	no	82
791	H7996xWV RIL		✓	yes	80
794	H7996xWV RIL		✓	no	26
795	H7996xWV RIL		✓	yes	57
798	H7996xWV RIL		✓	yes	69
801	H7996xWV RIL		✓	yes	82
807	H7996xWV RIL		✓	yes	40

*Percent wilting in response to *Ralstonia solanacearum* for parents (H7996, WV) and RILs. Scores are the average of 4-5 plants.

Table B3.3. Sequencing summary.

	Total paired reads	# of ASVs
Initial reads		NA
After adapter/primer clipping	6,898,036	NA
dada2 quality filtering	6,281,179	NA
Error correction, merged F and R reads, length filtering	5,684,371	34,201
Chimera removal	5,292,651	28,259
Non-target sequence removal	2,654,117	22,273
Likely contaminant removal	2,515,706	22,078
Low abundance filtering and filtering samples with <2000 reads	1,523,817	901
Outlier removal	1,416,614	901

***Table B3.4.** Table S4 Raw count table of all 901 ASVs across all samples.

***Table B3.5.** Sequencing sample metadata.

***Table B3.6.** Taxonomy table.

***Tables B3.4-6 are too large to be included in this document. They are available online at <https://apsjournals.apsnet.org/doi/suppl/10.1094/PBIOMES-02-20-0020-R> and are labeled Supplementary Tables 4, 5, and 6, respectively.**

Table B3.7. Differential abundance results from rhizosphere to endosphere, independent of genotype, phylum level.

Phylum	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Firmicutes	372.14832	-5.591804741	0.31747436	-17.613406	1.94E-69	3.11E-68
Deinococcus- Thermus	8.43553669	-33.68575868	2.39863499	-14.0437202	8.42E-45	6.73E-44
Bacteroidetes	220.012676	-0.983532573	0.17304793	-5.68358469	1.32E-08	7.03E-08
Verrucomicrobia	122.687651	0.989111341	0.24385228	4.05619072	4.99E-05	0.00019952
Chlamydiae	5.74750066	2.810104891	0.78449228	3.58206826	0.00034088	0.00109083
Nitrospirae	102.300609	0.958838314	0.27991668	3.42544185	0.0006138	0.0016368
Acidobacteria	68.3897953	0.837000406	0.27558996	3.03712229	0.00238849	0.0054594
Planctomycetes	46.3848637	1.302856937	0.45887298	2.83925399	0.00452191	0.00904383
Proteobacteria	7164.09436	-0.262347025	0.10370826	-2.52966378	0.01141719	0.02029722

Positive log2Foldchange means an increase in the endosphere compared to the rhizosphere, and vice versa for negative values.

***Table B3.8.** Differential abundance results from rhizosphere to endosphere, independent of genotype, family level.

***Tables B3.8 is too large to be included in this document. They are available online at <https://apsjournals.apsnet.org/doi/suppl/10.1094/PBIOMES-02-20-0020-R> and are labeled Supplementary Table 8.**

Table B3.9. Full differential abundance results for BB, rhizosphere to endosphere, family level.

Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Phylum	Class	Order
Blfidi19	11.26	-20.03	2.50	-8.02	1.02E-15	1.25E-13	Proteobacteria	Deltaproteobacteria	Myxococcales
Ardenticatenaceae	8.45	-27.16	4.17	-6.52	7.16E-11	4.37E-09	Chloroflexi	Anaerolineae	Ardenticatenales
Rubrobacteriaceae	25.52	-7.25	1.37	-5.28	1.27E-07	5.17E-06	Actinobacteria	Rubrobacteria	Rubrobacterales
Thermomonosporaceae	78.17	9.14	1.87	4.90	9.65E-07	2.94E-05	Actinobacteria	Actinobacteria	Streptosporangiales
Pseudonocardiaceae	265.16	4.19	0.88	4.77	1.81E-06	4.42E-05	Actinobacteria	Actinobacteria	Pseudonocardiales
Rhizobiaceae	246.12	1.68	0.37	4.50	6.76E-06	1.37E-04	Proteobacteria	Alphaproteobacteria	Rhizobiales
Mycobacteriaceae	23.19	-7.41	1.72	-4.31	1.63E-05	2.85E-04	Actinobacteria	Actinobacteria	Corynebacteriales
Cellvibrionaceae	74.47	3.22	0.84	3.81	1.38E-04	1.87E-03	Proteobacteria	Gammaproteobacteria	Cellvibrionales
Azospirillaceae	15.59	-7.45	1.94	-3.83	1.26E-04	1.87E-03	Proteobacteria	Alphaproteobacteria	Azospirillales
Xanthobacteraceae	370.98	-1.39	0.40	-3.51	4.49E-04	5.47E-03	Proteobacteria	Alphaproteobacteria	Rhizobiales
Bacillaceae	325.42	4.50	1.32	3.40	6.74E-04	7.47E-03	Firmicutes	Bacilli	Bacillales

Positive log2Foldchange means an increase in the endosphere compared to the rhizosphere, and vice versa for negative values.

Table B3.10. Full differential abundance results for UC82B, rhizosphere to endosphere, family level.

Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Phylum	Class	Order
Cryptosporangiaceae	39.82	-17.97	2.20	-8.15	3.56E-16	4.34E-14	Actinobacteria	Actinobacteria	Frankiales
Pyrinomonadaceae	39.72	-7.13	1.63	-4.38	1.18E-05	7.20E-04	Acidobacteria	Blastocatellia	Pyrinomonadales
TRA3-20	95.14	-2.10	0.51	-4.09	4.29E-05	1.74E-03	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales
Bacillaceae	325.42	5.07	1.42	3.58	3.44E-04	1.05E-02	Firmicutes	Bacilli	Bacillales
Rhizobiales	16.14	-5.70	1.83	-3.11	1.88E-03	4.59E-02	Proteobacteria	Alphaproteobacteria	Rhizobiales

Positive log2Foldchange means an increase in the endosphere compared to the rhizosphere, and vice versa for negative values.

Table B3.11. Full differential abundance results for MM, rhizosphere to endosphere, family level.

Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Phylum	Class	Order
Ilumatobacteraceae	28.24	-22.13	1.75	-12.63	1.37E-36	1.46E-34	Actinobacteria	Acidimicrobiia	Microtrichales
Geminicoccaceae	17.78	-20.05	1.75	-11.44	2.71E-30	1.45E-28	Proteobacteria	Alphaproteobacteria	Tistrellales
Bacillaceae	325.42	7.21	1.45	4.96	6.97E-07	2.49E-05	Firmicutes	Bacilli	Bacillales
Intrasporangiaceae	17.13	-6.65	1.64	-4.07	4.77E-05	1.28E-03	Actinobacteria	Actinobacteria	Micrococcales
Dongiaceae	26.63	-7.27	1.91	-3.82	1.35E-04	2.90E-03	Proteobacteria	Alphaproteobacteria	Dongiales
Beijerinckiaceae	69.33	-2.74	0.75	-3.66	2.57E-04	3.93E-03	Proteobacteria	Alphaproteobacteria	Rhizobiales
67-14	22.23	-6.69	1.81	-3.69	2.26E-04	3.93E-03	Actinobacteria	Thermoleophilia	Solirubrobacterales
Rhizobiales	16.14	-6.35	1.83	-3.46	5.37E-04	7.19E-03	Proteobacteria	Alphaproteobacteria	Rhizobiales
Iamiaceae	11.02	-5.28	1.62	-3.25	1.16E-03	1.38E-02	Actinobacteria	Acidimicrobiia	Microtrichales
Rubrobacteriaceae	25.52	-2.75	0.88	-3.12	1.83E-03	1.96E-02	Actinobacteria	Rubrobacteria	Rubrobacterales
Pirellulaceae	39.82	-4.02	1.33	-3.02	2.56E-03	2.40E-02	Planctomycetes	Planctomycetacia	Pirellulales
env.OPS_17	13.52	-6.47	2.16	-3.00	2.69E-03	2.40E-02	Bacteroidetes	Bacteroidia	Sphingobacteriales
Rhizobiaceae	246.12	1.18	0.40	2.97	2.96E-03	2.44E-02	Proteobacteria	Alphaproteobacteria	Rhizobiales
Solibacteraceae	12.06	-6.37	2.17	-2.94	3.33E-03	2.54E-02	Acidobacteria	Acidobacteriia	Solibacterales

Positive log2Foldchange means an increase in the endosphere compared to the rhizosphere, and vice versa for negative values.

Table B3.12. Full differential abundance results for Pearson, rhizosphere to endosphere, family level.

Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Phylum	Class	Order
Cryptosporangiaceae	39.82	-21.48	2.24	-9.60	8.22E-22	8.22E-20	Actinobacteria	Actinobacteria	Frankiales
Blfdi19	11.26	-16.94	2.73	-6.19	5.87E-10	2.94E-08	Proteobacteria	Deltaproteobacteria	Myxococcales
Bacillaceae	325.42	6.48	1.44	4.49	7.07E-06	2.36E-04	Firmicutes	Bacilli	Bacillales
Opitutaceae	22.38	-7.33	1.95	-3.76	1.67E-04	4.17E-03	Verrucomicrobia	Verrucomicrobiae	Opitiales
Polyangiaceae	23.74	-7.91	2.19	-3.61	3.02E-04	6.04E-03	Proteobacteria	Deltaproteobacteria	Myxococcales
Solibacteraceae	12.06	-7.03	2.17	-3.24	1.21E-03	2.01E-02	Acidobacteria	Acidobacteriia	Solibacterales
Xanthomonadaceae	154.91	1.95	0.64	3.02	2.49E-03	3.11E-02	Proteobacteria	Gammaproteobacteria	Xanthomonadales
Micrococcaceae	40.20	-2.68	0.87	-3.06	2.21E-03	3.11E-02	Actinobacteria	Actinobacteria	Micrococcales
Hyphomicrobiaceae	31.56	-3.35	1.17	-2.86	4.21E-03	4.68E-02	Proteobacteria	Alphaproteobacteria	Rhizobiales

Positive log2Foldchange means an increase in the endosphere compared to the rhizosphere, and vice versa for negative values.

***Table B3.13.** Full differential abundance results for CMII, rhizosphere to endosphere, family level.

***Tables B3.13 is too large to be included in this document. They are available online at <https://apsjournals.apsnet.org/doi/suppl/10.1094/PBIOMES-02-20-0020-R> and are labeled Supplementary Table 13.**

Table B3.14. Full differential abundance results for H7996, rhizosphere to endosphere, family level.

Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Phylum	Class	Order
Ardenticatenaceae	8.45	-32.55	3.82	-8.51	1.67E-17	1.47E-15	Chloroflexi	Anaerolineae	Ardenticatenales
Cellvibrionaceae	74.47	4.10	0.79	5.18	2.21E-07	9.72E-06	Proteobacteria	Gammaproteobacteria	Cellvibrionales
Nitrospiraceae	103.72	-2.46	0.64	-3.83	1.26E-04	3.68E-03	Nitrospirae	Nitrospira	Nitrospirales
Rhizobiaceae	246.12	1.18	0.35	3.42	6.32E-04	1.39E-02	Proteobacteria	Alphaproteobacteria	Rhizobiales
Bacillaceae	325.42	4.05	1.23	3.30	9.65E-04	1.70E-02	Firmicutes	Bacilli	Bacillales
Thermomonosporaceae	78.17	4.68	1.47	3.19	1.42E-03	2.08E-02	Actinobacteria	Actinobacteria	Streptosporangiales
Mycobacteriaceae	23.19	-3.94	1.27	-3.11	1.87E-03	2.35E-02	Actinobacteria	Actinobacteria	Corynebacteriales
Flavobacteriaceae	66.51	3.73	1.24	3.01	2.64E-03	2.90E-02	Bacteroidetes	Bacteroidia	Flavobacteriales
SC-I-84	136.19	-1.52	0.52	-2.93	3.42E-03	3.34E-02	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales
Caulobacteraceae	398.63	0.77	0.27	2.83	4.63E-03	3.71E-02	Proteobacteria	Alphaproteobacteria	Caulobacterales
Sphingomonadaceae	582.24	-0.86	0.30	-2.86	4.23E-03	3.71E-02	Proteobacteria	Alphaproteobacteria	Sphingomonadales
Microscillaceae	103.61	1.51	0.55	2.73	6.31E-03	4.62E-02	Bacteroidetes	Bacteroidia	Cytophagales

Positive log2Foldchange means an increase in the endosphere compared to the rhizosphere, and vice versa for negative values.

***Table B3.15.** Full differential abundance results for WV, rhizosphere to endosphere, family level.

***Tables B3.15 is too large to be included in this document. They are available online at**

<https://apsjournals.apsnet.org/doi/suppl/10.1094/PBIOMES-02-20-0020-R> and are labeled Supplementary Tables 15.

Table B3.16. Full differential abundance results for LA2093, rhizosphere to endosphere, family level.

Family	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Phylum	Class	Order
Ilumatobacteraceae	28.24	-20.22	1.69	-11.96	5.64E-33	5.64E-31	Actinobacteria	Acidimicrobiia	Microtrichales
Rhizobiaceae	246.12	2.60	0.37	6.97	3.07E-12	1.54E-10	Proteobacteria	Alphaproteobacteria	Rhizobiales
Bacillaceae	325.42	7.14	1.33	5.38	7.63E-08	2.54E-06	Firmicutes	Bacilli	Bacillales
Solirubrobacteraceae	50.22	-7.31	1.57	-4.66	3.14E-06	6.28E-05	Actinobacteria	Thermoleophilia	Solirubrobacterales
Rubrobacteriaceae	25.52	-6.42	1.37	-4.68	2.86E-06	6.28E-05	Actinobacteria	Rubrobacteria	Rubrobacterales
Pirellulaceae	39.82	-7.48	1.64	-4.57	4.81E-06	8.02E-05	Planctomycetes	Planctomycetacia	Pirellulales

Positive log2Foldchange means an increase in the endosphere compared to the rhizosphere, and vice versa for negative values.

***Table B3.17.** Average α -diversity in endosphere and rhizospheres of all tomato genotypes.

***Table B3.18.** Summary of relative abundance of taxa averaged at family level in all eight genotypes in each compartment.

***Tables B3.17-18 are too large to be included in this document. They are available online at <https://apsjournals.apsnet.org/doi/suppl/10.1094/PBIOMES-02-20-0020-R> and are labeled Supplementary Tables 17 and 18, respectively.**